REPORT DOCUMENTATION PAGE

AD-A175 105

JLE	1b. RESTRICTIVE MARKINGS NA
NA	3. DISTRIBUTION / AVAILABILITY OF REPORT Distribution unlimited

4. PERFORMING ORGANIZATION REPORT NUMBER(S) NYU Medical Center #8-1144-647	5. MONITORING ORGANIZATION REPORT NUMBER(S) NA

6a. NAME OF PERFORMING ORGANIZATION Dept. of Pathology, NYU MedCtr	6b. OFFICE SYMBOL (If applicable) NA	7a. NAME OF MONITORING ORGANIZATION Office of Naval Research

6c. ADDRESS (City, State, and ZIP Code) 550 First Avenue New York, NY 10016	7b. ADDRESS (City, State, and ZIP Code) 800 N. Quincy Street Arlington, VA 22217-5000

8a. NAME OF FUNDING / SPONSORING ORGANIZATION Office of Naval Research	8b. OFFICE SYMBOL (If applicable) ONR	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER N00014-83-K-0678

8c. ADDRESS (City, State, and ZIP Code) 800 Quincy Street Arlington, VA 22217-5000	10 SOURCE OF FUNDING NUMBERS			
	PROGRAM ELEMENT NO. 61153N	PROJECT NO. RR04108	TASK NO. NR 666-019	WORK UNIT ACCESSION NO.

11. TITLE (Include Security Classification)
Effect of Adjuvants on Response to Pneumococcal Polysaccharide Injected Intraperitoneally Platelet-Derived Immunoregulatory Activity.

12. PERSONAL AUTHOR(S)
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13a. TYPE OF REPORT Final	13b. TIME COVERED FROM 7/1/83 TO 8/31/86	14. DATE OF REPORT (Year, Month, Day) 12/11/86	15. PAGE COUNT 48

16. SUPPLEMENTARY NOTATION
NA

17.	COSATI CODES		18 SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	detoxified endotoxin, platelet factor 4, immunoaugmentation, IgD, adjuvants, pneumococcal polysaccharide
08			

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

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20. DISTRIBUTION / AVAILABILITY OF ABSTRACT ☑ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	21. ABSTRACT SECURITY CLASSIFICATION (U)
22a NAME OF RESPONSIBLE INDIVIDUAL Dr. J.A. Majde	22b. TELEPHONE (Include Area Code) (202) 696-4055	22c. OFFICE SYMBOL ONR

DD FORM 1473, 84 MAR 83 APR edition may be used until exhausted. SECURITY CLASSIFICATION OF THIS PAGE
All other editions are obsolete.

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Summary

A platelet-derived factor was described which reverses both antigen specific and nonspecific (Con A induced) suppression of antibody production in mice. The factor was identified as a granule derived platelet factor 4 (PF4). Immunoregulatory activity of PF4 was found to be dependent on the presence of a proteolytic enzyme during its release from platelets. Both mouse and human PF4 from platelet releasate and from serum are absorbed by a T cell subset from mouse lymph node and spleen, but not from thymus, with surface characteristics of suppressor T cells. In addition, Con A induced splenic suppressor cells adhere to dishes coated with PF4. However, PF4 or platelet releasate do not reverse Con A induced suppression in vitro. In view of the known tendency of PF4 to adhere to heparan sulfate in the blood vessel wall, it is suggested that PF4 changes the distribution of suppressor cells in vivo, thereby interfering with their activity.

Various adjuvants were examined for their ability to affect the serum antibody response to pneumococcal polysaccharides (pps) types 3 and 14. Detoxified endotoxin, (D=LPS), lectins and lymphokine mixtures all enhance the response to pps when injected 2-4 days after pps. Coupling of MTP to pps renders it more immunogenic, as does emulsification of the pps with Nor-MDP in squalene arlacel. IgD injected together with pps 14, or 1 week before, enhances both primary and secondary responses, particularly to subimmunogenic doses. Low and high dose tolerance to pps antigens are reversed by D-LPS, whereas low (but not high) zone tolerance to both pps 3 and 14 is reversed by PF4. Low zone tolerance is not observed in aged or nu/nu mice. Dose response curves for pps types 3 and 14 differ by 100 fold. The effect of combination of agents which reverse T cell suppression and which stimulate B cell activation, should be examined.

G. J. Thorbecke, M.D.

Platelet-Derived Immunoregulatory Activity

When SJL mice are injected intravenously (iv) with γ-irradiated syngeneic lymphoma (γ-RCS) cells at the same time as sheep erythrocytes (SRBC) a marked suppression of the ensuing plaque forming cell (PFC) response in the spleen is observed [Katz et al., J. Natl. Cancer Inst. 72:125, 1984]. This suppression has been interpreted as due to the strong T cell proliferation [Hayama et al., J. Natl. Cancer Inst. 72:321, 1984], IL-2 [Hayama et al., Cell. Immunol. 79:134, 1983] and IFN-γ [Ponzio et al., J. Natl. Cancer Inst. 72:311, 1984] production induced by γ-RCS both in vivo and in vitro. Cyclophosphamide pretreatment prevented the induction of this suppression, suggesting that a suppressor T cell--mediated phenomenon might be involved.

Immunoregulation in vivo is sometimes accomplished by the injection of monoclonal antibodies or immune sera. In attempting to apply this approach in the elucidation of the mechanism of γ-RCS induced suppression, we obtained an unexpected immunoregulatory effect with iv injected normal mouse serum (NMS), which had been intended to serve as a control. A further analysis of this phenomenon showed that absorption with normal spleen cells of any mouse strain removed the immunoregulatory factor from NMS, confirming its complete lack of strain specificity. As will be shown below we have further characterized this factor and found that it is non-dialyzable and regularly present in normal mouse and human serum, but not in plasma. This factor counteracts suppression when injected 1 day prior to antigen, frequently enhancing the response to SRBC above control levels, and is present in the supernatant of clotted mouse or human platelets as well as in human platelet lysates.

As shown in Table 1, injection of γ-irradiated RCS simultaneously with SRBC into SJL mice caused profound suppression of the PFC response in the spleen measured 4 days later, as described before. This effect was completely abolished by iv injection of 0.2 ml of a 1:4 dilution of NMS. The two experiments in Table 1 represent typical experiments, one in which the response was brought back to the control level by injection of NMS (Expt. 1) and one in which the NMS caused the response to become higher than in the control mice injected with SRBC alone (Expt. 2). Both syngeneic and allogeneic NMS caused the effect, and serum from 9-months--old had the same effect as serum from 2-months-old SJL mice. Absorption with unrelated (A/J mouse) spleen cells removed the activity from the NMS, while absorption with nu/nu (BALB/c) spleen cells did not.

The results in Table 2 confirm these findings and show that the same phenomenon is observed in BALB/c mice using BALB/c NMS. Again, absorption with BALB/c spleen removed the nonspecific enhancing activity, but absorption with nu/nu spleen cells did not. Although the degree of suppression obtained in BALB/c mice, either with γ-RCS (not shown) or with normal LN cells, was only moderate (~ 30%), both the suppression and the reversal to normal or above normal responses after NMS injection were statistically significant. Even serum obtained from a single mouse, clotted, spun, and reinjected into the same mouse caused the phenomenon of enhancement (Expt. 1, Table 2: autologous serum).

The results in Table 3 show that normal human serum (NHS) also had the immunoregulatory effect in the response of mice to SRBC. In two experiments (Expts. 1 and 2) the response was much higher than in controls not injected with serum or γ-RCS, but in Expt. 3 the response only went

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back to the control level, overcoming the γ-RCS induced suppression. Absorption of the NHS with mouse spleen or cells from a cytotoxic T cell line reduced or abolished the "contrasuppressive" effect of the NHS. The results in Table 3 suggest, in addition, that the effect is due to a non-dialyzable, heat-resistant factor.

It was of interest to determine whether NHS would also affect the response to other antigens. We, therefore, performed an experiment in SJL mice with 10 μg TNP-Ficoll, to see the effect on a relatively thymus-independent response. The response was not much affected by injection of γ-RCS simultaneously with antigen, as shown previously, but it went from 43,650   1.05 to 93,100   1.1, when 0.05 ml NHS was injected iv on the day before antigen (Table 4).

Another approach to the identification of the factor causing this effect was by use of proteolytic enzyme inhibitors. Addition of soybean trypsin inhibitor (10 μg/ml) or of tranexamic acid (50 μM) prior to clotting of blood resulted in NMS devoid of the effect in vivo (Fig. 1). Similarly 25μM amino-n-caproic acid (EACA) added to blood prior to clotting prevented the appearance of the factor in NMS, while addition of EACA to serum after clotting had no effect (Fig. 2). Plasma failed to affect the immune response in vivo, and, more significantly, clotting of plasma produced NMS which also lacked the effect (Figs. 1 and 2). The results in Fig. 1 show, in addition, that the effect of NMS can be seen in the absence of a suppressor cell inducing agent, since the mice in that experiment did not receive any γ-RCS injection.

In view of the lack of effect of NMS prepared from plasma in the absence of cells (Fig. 2) the results suggested that the factor was somehow generated from peripheral blood cells during clotting. Although interaction between B cells and plasminogen have been described the first cells we turned our attention to were the platelets. The results in Fig. 2 show that the supernate from platelets, which had been spun down from lymphocyte and erythrocyte-depleted whole mouse blood and which were then allowed to clot by addition of thrombin (1 U per ml), significantly reversed suppression induced by γ-RCS (Fig. 3). The results in Table 5 demonstrate that similar results were obtained with releasate from human platelets incubated with thrombin. The releasate greatly enhanced the response to SRBC (Expt. 1), at least as much as was observed with serum. Identification of lymphoid cells capable of binding the factor:

Further studies established that serum and platelet releasate of both human and mouse origin can prevent the suppression of the antibody (PFC) response to sheep erythrocytes (SRBC) induced either by syngeneic γ-irradiated lymphoma cells in SJL mice or by concanavalin A (con A) in all mouse strains tested. In many cases the response of mice injected with an appropriate amount of platelet releasate is above that of control, unsuppressed mice. Since splenic T cells could absorb the factor from serum, we attempted to characterize the cells in the spleen responsible for this absorption. It was found that helper cells (ly1+,L3T4+) cells are not needed for this effect, whereas ly2+ cells are. The other cell surface markers found on the absorbing cells are Qa1,Ly22,Ly6,Qa4 and Qa5; suggesting that activated suppressor T cells are involved in removing the platelet factor.

Methods used in these experiments were described in the published papers by Katz et al (J. Immunol. 134:3199, 1985; Cell. Immunol. 100:57, 1986; Proc. Natl. Acad. Sci. 83:3491, 1986.) and in the footnotes to the tables. The antibodies used for killing of subpopulations in the

presence of non-toxic rabbit C were: GK1.5 (rat IgG2b anti-L3T4); 19/178 (mouse IgG2a anti-Lyt2.2); SK70.94 (mouse IgG2a anti-Ly-m6.1E); T28.45.9 (mouse IgG2b anti-Ly-m 22.2); 2-2.1 (mouse IgG2b anti-Lyt1.2); B16-146 and B16-147 (mouse IgM anti-Qa4 and 5); and allo antiserum to Qa1.

The results can be seen in Tables 6 and 7 and in Figs 4A, B, and C. Table 6 shows examples of the reversal of con A-induced suppression by platelet releasate or serum in $CB6F_1$ mice. Although the effect of 50 µg con A was not completely reversed, that of 5 to 10 µg con A was and, in some cases, the control response to 2×10^7 SRBC alone (injected ip) was much lower than the response in mice receiving platelet factor and con A as well as antigen. In addition, the results of Expt 4 show that the serum could be injected before con A or 1 day after con A (just prior to antigen) with similar results.

The results in Table 7 show that spleen or lymph node cells absorb the suppression reversing effect from serum, while thymus cells do not. Two absorptions with 10^8 spleen cells per 0.5 ml of serum were sufficient to remove all activity, while two absorptions with 2×10^7 cells resulted in a greatly reduced activity.

The results in Fig 4A show that killing the $Lyt2^+$ (2.2^+) cells in spleen cells removes their ability to absorb, while removal of $GK1.5^+$ cells ($L3T4^+$) has no effect. The results in Figs 4B and C show that removal of the high $Lyt1^+(1.2^+)$ bearing cells which can be killed by antibody +C has little effect on the ability of spleen cells to remove the activity while removal of $Qa1^+$, $Qa4^+,Qa5^+,1y6.1E^+$ or $Ly22^+$ cells in each case abolishes absorbing activity.

Identification of the immunoaugmenting platelet factor as platelet factor 4.

Characterization of the factor responsible for the immunoregulatory activity was greatly facilitated by the observation that serum from a patient with gray platelet syndrom does not contain the factor, suggesting that it is an α granule component. Moreover, heparin agarose absorbs the activity and purified platelet factor 4 (PF4) exhibits similar immunoregulatory activity as the whole platelet releasate. Other known constituents of α granules, such as platelet derived growth factor (PDGF) and low affinity (LA) PF4 have little or no activity. In addition, activity in human serum is neutralized by goat anti-human PF4.

Radioimmuno assay kits were used for assays of LAPF4 and PF4 (Amersham and Abbott Labs). Lactic dehydrogenase (LDH) and β-glucuronidase were assayed with kits from Sigma Chemical Co. Heparin-neutralizing activity was measured by the method of Harada and Zucker (Thromb. Diath. Haemorrh. 25:41,1984). Serotonin release was measured in platelets that had been incubated with 0.5 µM ^{14}-C-serotonin (Jerushalmy and Zucker, Thromb. Diath. Haemorrh. 15:413,1966) (56mCi/mMol. Amersham). Heparin agarose was purchased from Pierce Chem. Co.

Results are summarized in Tables 8-10, Figs. 5A and B, and Fig. 6.

Figs. 5A and B show dose-response curves of immunoregulatory activity obtained with serum (Fig. 5A) and with platelet releasate (Fig. 5B). The greatest effect was obtained with 0.2 ml of undiluted serum, but even 0.2 ml of 1:10 diluted serum increased the number of PFC above the control value. With releasate the greatest effect was noted after injection of material released from about 3×10^6 platelets, and releasate from 10^6 platelets are still effective.

Fig. 6 shows the simultaneous release of immunoregulatory activity and the known α-granule constituents: PF4, serotonin and β-glucuronidase

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by different concentrations of thrombin added to a human platelet suspension and incubated for 60 minutes. LDH was measured as an indication of platelet lysis, since it is not present in α granules and should not be released by thrombin. Its concentration in the releasates was less than 8°/o of its concentration in the platelets (as measured after completely lysing the platelets).

Tables 8-10 show the data which suggest that the immunoregulatory activity is due to PF4. The purified PF4 and LAPF4 preparations tested (Table 9) were gifts from Dr. S. Niewiarowski (Temple Univ., Philadelphia) and were isolated according to Varma et al (Biochim. Biophys. Acta 701:7,1982). Both the binding to heparin and the neutralization of the activity by anti-PF4 support the finding that isolated PF4 has the activity.

The effect of 0.2-0.6 µg, PF4 was as great as that of the releasate from 2×10^7 platelets (Table 9), which is in agreement with the observation that platelets contain .18 µg of PF4 per 10^7 platelets (Files et al., Blood, 58:607, 1981). The efficiency of such small amounts of this immunoaugmenting protein is remarkable and also agrees with the effectiveness of 50 µl of serum, since human serum contains approximately 5 µg PF4 per ml (Lonky and Wohl, J. Clin. Invest. 67:817,1981).

Presence in fetal calf serum of material able to reverse Con A-induced suppression of antibody production in vivo.

It was established that fetal calf serum, like human and mouse serum, reversed Con A induced suppression in vivo (Table 11). One intravenous injection of 0.2 ml 1:4 diluted serum from all three species, one hour prior to Con A, completely overcame the Con A induced suppression of the response to SRBC injected the next day and even raised responses above those in control mice not injected with Con A. Absorption with mouse spleen cells removed this activity from mouse and human sera, in agreement with previous observations, and from fetal calf serum as well.

Inability of platelet releasate to alleviate Con A-induced suppression in vitro.

In order to avoid the presence of PF4 in the culture system, absorbed fetal calf serum was then used in experiments on the immune response of lymph node cells (which unlike spleen are devoid of platelets) to TNP-B.abortus in vitro. The results in Table 12 show that added platelet releasate (approx. 20 µg PF4 per dish) had no effect. Addition of Con A to these lymph node cell cultures caused dose-related suppression, with 12-29°/o of the response remaining in the presence of Con A. The suppression in the presence of platelet releasate was essentially the same, with 15-34°/o of the response remaining. Thus, there was no evidence that the factor in platelet releasate which prevents suppression in vivo could function similarly in vitro.

Fractionation of Con A-induced suppressor cells by adherence to PF4-coated surfaces

The results in Table 13 represent two series of 3 experiments each in which spleen cells taken from mice one day after injection of Con A (containing suppressor cells) were added to normal spleen cells that were responding to SRBC added simultaneously. Attempts were made to fractionate the suppressor cells induced by Con A by adherence to PF4-coated beads or petri dishes. In the first series of experiments the cells were slowly rotated at 4°C with heparin-agarose beads which had adsorbed PF4 from human platelet releasate. The beads were carefully removed from the

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nonadherent cells and gently washed (2x) before being vigorously tritur-
ated to retrieve adherent cells. About 10°/° of cells was recovered in
the adherent cell fraction. A similar yield of cells was obtained with
control heparin-agarose beads. The suppressor cell effect, however, was
present only in the PF4-adherent fractions and was depleted from the
PF4-nonadherent fractions.

In the second series of experiments (Table 3) we used PF4 from mouse
serum to coat petri dishes. A PF4-free human plasma fraction was used in
the diluent fluid, which was also used to coat control dishes. The sup-
pressor cells were fractionated by adherence to such coated dishes. Any
cells even loosely associated with the bottom of the dishes were consid-
ered "adherent." Prior to fractionation by adherence to PF4-coated
dishes, the cells were allowed to adhere at 37°C for 1 hour to culture
dishes in order to remove macrophages in two of the three experiments.
This did not appear to influence the results. Again, as in the first
series of experiments, the suppressor cells were found in the PF4-ad-
herent fraction. Results from a representative experiment are shown in
Fig. 7. When both series of experiments were taken together there was a
statistically significant difference between the responses obtained in
dishes with PF4-adherent and nonadherent cells ($p=.034$). In contrast,
after control incubation, the suppressor cells were evenly distributed
between adherent and nonadherent fractions ($p=0.93$).

Immune enhancement of the response to pneumococcal polysaccharides (pps)

The aim of these experiments was to develop methods of immunization
with pneumococcal polysaccharides which are more effective than the
existing ones. Although under normal conditions a healthy young adult
will respond with satisfactory protective antibody production to immuni-
zation with the multi-polysaccharide pneumo vaccine, immunodepressed in-
dividuals, such as is for instance the case with aged people, do not
(Ammann et al., Proc. Soc. Exp. Biol. Med. 164:312, 1980; Schwick and
Becker, in Current Problems in Immunology, Bayer Symposium 1:253, 1969).

Effect of lectins and lymphokines:

Initial experiments were performed with lectins (Concanavalin A and
PHA-P) given 2 days after antigen, following the procedure described for
pps type 3 by Taylor et al. (Cellular Immunology 83:26, 1984). The
results in Table 14 illustrate this effect. It is possible to replace
the lectins by injection of a crude mixture of lymphokines, particularly
high in IL-2, and also containing some TRF-like activity, colony
stimulating factors and IL-3, prepared either by PHA stimulation of the
LBRM-33 cell lines in serum free medium or by incubation of SJL lymph
node cells with syngeneic lymphoma cells [Hayama et al., Cell. Immunol.
79:134, 1983].

Preparation of the N-acetyl-muramyl-L-alanyl-D-isoglutaminyl-L-ly-
sine (MTP) conjugate was done by Drs. Baschang and Hartmann (Ciba Geigy,
Basel, Switzerland). They dissolved 43 mg pps type 3 and 40 mg MTP in 5
ml phosphate buffer (pH 7.0) by gentle warming to 40°C and stirring. To
the viscous solution at room temperature was added 80 mg of N-ethyl-N'--
(3-dimethyl amino propyl)-carbo-diimide-HCl. The mixture was stirred for
15 hrs. and extensively dialyzed against 0.2M NaCl and then against wat-
er, after which it was lyophilized to yield 40.7 mg. of conjugate The
content of MTP as determined by the Morgan-Elson-reaction was 25 µg
MTP-mg conjugate (2.5°/°). It was still soluble in water up to
0.5°/°, although highly viscous.

Immunization with this MTP conjugate of pps type 3 has shown that its immunogenicity is enhanced by this coupling procedure (Fig. 8). Both serum titers and PFC/spleen are higher after injection of the conjugate than after injection of the same pps alone or mixed with uncoupled MDP.

Injection of lectins on day 2 after the pps type 3-MTP conjugate further enhanced the response detected on day 5 and caused the appearance of 7S serum antibody by Day 10 which was not detected after immunization with the conjugate alone at this time. Even after immunization with the unconjugated pps, although not raising serum antibody titers very much, injections of lectins caused the induction of detectable 7S antibody by Day 10. Thus, the combination of these two adjuvants effects was more marked than either one alone (Table 15).

Effect of Primary Dose of pps on Primary and Secondary Responses:

In view of the known antigen dose responsiveness to pps type 3 in mice, it seemed of interest to compare dose effects for types 14 and 3 in more detail. The results in Tables 16 and 17 show the dose response relationship for types 3 and 14 pps in BALB/c mice. While the optimal immunizing dose for pps 14 was found to be 25 μg, the best primary responses for pps 3 were obtained with 0.1μg. In addition, higher doses of pps 3 caused unresponsiveness, as seen both during the primary response and after boosting with killed pneumococci. In the case of pps 14, the secondary response was slightly higher than the primary response to killed bacteria when priming had occurred with 25 μg, but lower than the primary response when priming was done with 0.2 to 0.5 μg. In contrast, the response to type 3 was never higher in the secondary response than in the primary response, whether boosting was done with whole bacteria or with 0.1 μg pps 3. Rather, the secondary response tended to be somewhat lower than the primary response, particularly after priming with very low (0.002 to 0.005 μg) or high (5μg) doses.

Similar results were obtained in (CB6)F1 mice in which 5 μg pps 3 caused complete unresponsiveness, while 0.1 μg caused optimal immunization. Killed bacteria also caused unresponsiveness when injected in doses of 10^9, whereas 10^8 bacteria immunized. Immunization with 0.1 μg in athymic BALB/c mice resulted in lower serum titers than in either euthymic BALB/c or (CB6) F_1 mice. A dose of 5 μg induced unresponsiveness in athymic mice as well as in euthymic mice, suggesting a direct effect on B cells of the supraoptimal antigen dose (Table 18).

It had been reported by Baker and coworkers that injection of very low doses of pps 3 caused the appearance of suppressor T cells, which was best demonstrated by challenging with an optimal immunizing dose 3 days after the tolerizing injection. The results in Table 10 and Fig. 9 show that this can readily be demonstrated for both pps 3 and pps 14. The tolerizing dose for pps 3 was in the range of 0.002-0.005 μg, whereas with pps 14 0.2 μg proved most effective. This effect is totally antigen specific (Fig. 9) and absent in athymic mice (Table 20), suggesting that it is mediated by T cells.

Response of aged and of athymic mice to pps:

Primary responses to both pps 14 and 3 were not significantly lower in aged than in young adult mice (Tables 16 and 20). In fact, there was a tendency to slightly higher 7S antibody responses in aged mice to pps type 3. It was of interest to determine whether aged mice were also susceptible to low dose tolerance. The results in Table 5 show that the response to 0.1 μg pps 3 is not significantly affected by injection of

0.005 µg of this pps 3 days earlier, suggesting that aged mice are re-
sistant to this form of suppression.

Absence of suppressor cell induction in aged mice may explain the
higher secondary response seen in aged as compared to young mice to pps
14 (Table 16). However, although Nu/Nu mice do not exhibit low dose tol-
erance, their secondary response to pps 14 is low, particularly for the
IgG component (Table 16). It appears, therefore, that some degree of T
helper or augmenting function is involved in the secondary response to
pps 14, even in aged mice.

Effect of endotoxin on response to pps:

The results in Table 18 show that injection of 10 µg detoxified
endotoxin (D-LPS; Ribi Immunochem. Res., Inc.) 4 days after injection of
5 µg pps reverses the high dose induced unresponsiveness. Low titers
were still detected in sera taken on day 5, but sera taken on days 10 and
15 showed titers similar to those induced by 0.1 µg pps 3. It was,
therefore, obvious that D-LPS could have a dramatic effect on antibody
responses to pps 3 even as late as 4 days after antigen.

The effect of D-LPS on the response to optimal immunizing doses of
pps was next examined (Table 21). Simultaneous injection with 25 µg pps
14 caused little effect, but injection 2-4 days after the antigen en-
hanced both primary and secondary responses. This effect was most sig-
nificant when D-LPS was injected on day 3 and was evident in both IgM and
IgG antibody responses. The secondary responses to killed bacteria in-
jected on day 15 stayed higher throughout day 10 after the boost (Day 25)
and caused a higher IgG antibody component than the response induced by
bacteria in unprimed mice (see Table 1) or in mice primed with pps 14
without D-LPS (Table 21). This effect of D-LPS, however, appeared to re-
quire T cells, since it was absent in athymic mice. Injection of D-LPS 3
days after administration of 0.1 µg pps 3 also caused augmented primary
responses with 7S titers as high as 5.5 ± 0.5 on days 10 and 15.
However, priming for a secondary response to killed bacteria was still
not obtained for pps 3 (data not in tables).

The effect of D-LPS on low dose tolerance was also examined (Table
22). It was found that injection of D-LPS, either on the same day as the
tolerizing or on the same day as the immunizing injection of pps, par-
tially reversed the suppression, both for pps 14 and for pps 3. Other
agents which are known to affect antibody responses in vitro and in vivo
and to cause B cell proliferation are 8-mercaptoguanosine and 8-bromo-
guanosine. As previously described for tolerance to deaggregated human
Ig in mice, injection of 8-bromoguanosine (5-10 mg) at the time of the
immunizing dose of pps 3 also caused a partial reversal of the tolerance
induced by a prior low dose of pps (data not shown). However, higher
doses of these agents were needed to significantly enhance the primary
response to optimal antigen doses. Table 23 illustrates the effect of
8-mercaptoguanosine on the primary response to 25 µg pps 14. A single
dose of 30 mg of this drug on day 0 caused a significant increase of the
19S and 7S responses, but also killed approximately 50°/° percent of
the mice.

Response to emulsified pps:

Injection of MTP or Nor-MDP in saline simultaneously with optimal
immunizing doses of pps, failed to raise the responses significantly (not
shown). Admixture of Nor-MDP in a water in oil emulsion of pps 14 or pps
3 prepared with squalene/arlacel caused a higher response than was seen
without Nor-MDP, including a higher 7S antibody response (Table 24). A

similar effect of Nor-MDP was observed when the dose of pps 3 per mouse
was 1μg (not shown). No such effect was obtained when a mixture of the
Syntex adjuvant pluronic/arlacel was used to prepare the emulsion with
the pps (Table 24) (Pluronic=Surface-active copolymers of hydrophilic and
hydrophobic components, described by Hunter and Bennett, J. Immunol.
133:1, 1984). The 7S antibody responses in all the animals immunized
with pps in emulsions decreased by day 21. There was no evidence, there-
fore, that administration of the pps in such emulsions induced a more
sustained response than did injection of the pps in saline.

Effect of IgD on the Immune Response to pps:

In previous studies it was shown that increased antibody responses
were obtained in mice pretreated with IgD injections one week prior to
the primary antigen injection (Xue et al., J. Exp. Med. 159:103, 1984;
Coico et al., Nature 316:744, 1985). This effect was mediated by the in-
duction of IgD receptors on the majority of L3T4$^+$, lyl$^+$, Ly2$^-$ T
cells (Coico et al., J. Exp. Med. 162:1852, 1985). The results in Fig.
10 show the effect of pretreatment with IgD 1 week prior to immunization
(Day -8, -7 and -6) and of simultaneous injection of IgD with antigen
(Day 0) on primary and secondary responses to pps 14. There was a small
augmenting effect on the response to the optimal immunizing dose, which
was more clearly seen during the secondary (p<.05) than during the prim-
ary response (day 10:p=.01) and affected both 7S and 19S responses. The
primary response to 5 μg pps 14 was similarly augmented, again more by
pretreatment with IgD than by simultaneous injection of IgD with anti-
gen. However, even injection of IgD on day 0 significantly enhanced the
primary response to the low dose, 0.2 μg pps 14 (p=.02 day 5; p=.001 day
15; p<.05 day 25). When comparison is made with the results in Table 16,
it can be seen that the secondary response to killed bacteria, injected 2
weeks after 0.2 μg pps 14 + IgD, by day 10 after boosting (day 25) was
higher than the primary response to the killed bacteria used for the
challenge. Thus, it appeared that, in the presence of IgD, even 0.2 μg
of pps 14 was able to cause a slight degree of priming.

The effect of pretreatment with IgD on the response to pps 3 was
next examined. IgD given together with a supra optimal dose (10 μg pps
3) did not prevent tolerance induction e.g. serum titers in such mice
after challenge with killed bacteria were below detectable levels (log$_2$
<2.5). as in mice injected with 10 μg pps 3 without IgD (not shown). IgD
also had no detectable effect on the response to an optimal immunizing
dose of pps 3 (0.1 μg). The secondary response to killed bacteria in-
jected 10 days after the primary injection of pps 3 was only slightly in-
creased in the IgD treated mice (by day 20; data not shown).

Effect of Platelet Releasate on Antibody Response to Pneumococcal
Polysaccharides (pps): Injection of platelet releasate simultaneously
with a primary dose of pps 14 (Fig. 11) or pps 3 (data not shown), had no
effect on the magnitude of the primary response. However, the secondary
response of both 19S and 7S antibody to killed pneumococci injected 2
weeks after the primary injection of pps was higher in the groups of mice
which had received weekly injections of platelet releasate (Fig. 11).
Although on days 20-25 there was little difference between the primary
response to killed bacteria and the response to bacteria injected in
previously immunized mice, the response was maintained (see day 30) at
higher levels in the pps primed mice. Mice primed with pps and given a
single injection of platelet releasate on day 0, showed a significantly
enhanced secondary response (5-15 days after the boost: p=0.05 for days

20 and 25, p <0.01 for day 30). A similar effect was observed when the mice received mouse serum instead of platelet releasate (data not shown).

To determine whether the enhanced responses were due to inhibition of suppression, an injection schedule of pps was employed which induces low dose tolerance and/or suppressor T cells. Mouse serum and PF4 isolated from human serum were used as the immunoregulatory agents in these experiments (Figs. 12 and 13). As shown above, ip injection of 0.2 μg of pps 14 3 days prior to an immunizing dose of 25 μg significantly reduced the primary response. The low dose tolerance was reflected in lower 19S and absent 7S responses on days 5 and 10. Either mouse serum or PF4, injected at the same time as the suppression-inducing dose, partially or completely prevented the low dose tolerance (Fig. 12).

Similarly, ip injection of 0.005 μg o. pps 3 three days prior to an optimal immunizing dose of 0.1 μg greatly reduced the primary response (Fig. 13). Injection of mouse serum on day -3, but not on day 0, partially overcame the effect of the pretreatment with the low dose (p<.01 on day 5), while PF4 restored the response virtually completely to that in mice injected with 0.1 μg pps 3 alone (difference from control p=.001 on day 5).

As shown above PF4 prepared from APMSF-treated platelets had no detectable immunoregulatory activity on the Con A induced suppression. The results in Table 25 show that similar findings were obtained with respect to the prevention of low dose tolerance to pps 3. Injection of PF4 prepared from normal platelet releasates prevented low dose tolerance when injected on day -3 together with the low antigen dose, whereas APMSF-PF4 did not. Since any remaining APMSF had been dialyzed out, this was not due to a toxic effect of the enzyme inhibitor.

Thus PF4 isolated from human or mouse serum by adherence to and elution from heparin agarose prevents not only Con A-induced suppression in vivo, but also antigen specific suppression induced by low doses of pps. The observation that suppressor cells can be removed from spleen cell suspensions by adherence to dishes or beads coated with PF4 derived from mouse serum or from human platelet releasate suggests that suppressor cells have an affinity for PF4. Since PF4 has no effect on Con A induced suppression of the PFC response in vitro, we have postulated that it causes a redistribution of the suppressor cells in vivo that interferes with their activity in vivo..

Although the primary response to an optimal immunizing dose of pps is not affected by PF4, the secondary response induced 2 weeks later is higher when PF4 is administered at weekly intervals during the response. Perhaps this means that counteracting suppressor cells induced during the primary response will allow better priming to pps for a secondary response, but further studies are needed to analyze this possibility.

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Thorbecke, G. Jeanette, and Zucker, M.B. 1986. Composition and method for restoring suppressed immune responses. Serial No. 794,105 (pending).

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TABLE 1

ANTIBODY PRODUCTION ENHANCING AND/OR SUPPRESSION REVERSING EFFECT OF NORMAL MOUSE SERUM IN SJL

Injected IV on			Expt. 1	Expt. 2
Day 0 SRBC (10^7)	Day 0 γ-RCS (2×10^7)	Day -1 NMS	PFC / Spleen	PFC / Spleen
+	-	-	11,700 ×÷ 1.2	8,100 ×÷ 1.1
+	+	-	2,300 ×÷ 1.4 *o	1,100 ×÷ 1.1
+	+	OLD SJL(UNABS.)	11,500 ×÷ 1.2^o	N.D.
+	+	YOUNG SJL(UNABS.)	10,500 ×÷ 1.1	28,200 ×÷ 1.1
+	+	B10. X 9R(UNABS.)	N.D.	N.D.
+	+	OLD SJL(ABS. WITH A SPLEEN)	4,700 ×÷ 1.4*	1,100 ×÷ 1.1
+	+	B10 X 9R(ABS. WITH A SPLEEN)	N.D.	34,700 ×÷ 1.1
+	+	B10 x 9R(ABS. WITH NUDE BALB/c SPLEEN)	N.D.	

\# RESULTS ARE EXPRESSED AS GEOMETRIC MEAN ×÷ SE (N=5) OF PFC PER SPLEEN ASSAYED 4 DAYS AFTER IV INJECTION OF SRBC.

* N.S.

o $P < .01$.

TABLE 2

ANTIBODY PRODUCTION ENHANCING AND/OR SUPPRESSION REVERSING EFFECT OF NORMAL MOUSE SERUM IN BALB/c MICE#

Injected IV*			Expt. 1	Expt. 2
Day 0 SRBC	Day 0 SJL Cells	Day -1 BALB/c Serum	PFC / Spleen	PFC / Spleen
+	-	-	$15{,}800 \times\!\!\div 1.1$	$21{,}900 \times\!\!\div 1.0$
+	+	-	$10{,}700 \times\!\!\div 1.1$ ° **	$14{,}800 \quad 1.2^{+}$ ⊕
+	+	+ UNABSORBED (SYNGENEIC)	$39{,}800 \times\!\!\div 1.1$ **	$20{,}400 \times\!\!\div 1.0^{+}$
+	+	+ ABS. WITH BALB/c SPLEEN	$11{,}500 \times\!\!\div 1.1$ °	$15{,}100 \times\!\!\div 1.2$ ⊕
+	+	+ ABS. WITH NUDE BALB/c SPLEEN	$40{,}700 \times\!\!\div 1.1$	$31{,}600 \times\!\!\div 1.1$
+	+	+ UNABSORBED (AUTOLOGOUS)	$44{,}700 \times\!\!\div 1.1$	N.D.

* NORMAL SJL LYMPH NODE CELLS (2×10^{7}) INJECTED IV TOGETHER WITH SRBC (5×10^{6}, EXPT. 1; 10^{7}, EXPT. 2).

** $p < .0001$.

° AND ⊕ N.S.

\+ $p < .002$.

\# RESULTS ARE EXPRESSED AS GEOMETRIC MEAN $\times\!\!\div$ SE (N=5) OF PFC PER SPLEEN ASSAYED 4 DAYS AFTER IV INJECTION OF SRBC.

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TABLE 3

EFFECT OF HUMAN SERUM ON THE ANTI-SRBC RESPONSE OF SJL MICE

| Injected iv | | | | | |
Day 0 SRBC (10^7)	Day 0 γ-RCS (2×10^7)	Day -1 Serum (50 µl)	Expt. 1 PFC/Spl	Expt. 2 PFC/Spl	Expt. 3 PFC/Spl
+	-	-	$9{,}100 \overset{\times}{\div} 1.0$	$7{,}950 \overset{\times}{\div} 1.1$	$6{,}100 \overset{\times}{\div} 1.1$
+	+	-	$1{,}500 \overset{\times}{\div} 1.2$	$1{,}100 \overset{\times}{\div} 1.3$	$1{,}300 \overset{\times}{\div} 1.1$
		Human (NHS)			
+	+	Fresh	$26{,}900 \overset{\times}{\div} 1.1$	$17{,}400 \overset{\times}{\div} 1.1$	$7{,}250 \overset{\times}{\div} 1.2$
+	+	Absorbed**	$9{,}500 \overset{\times}{\div} 1.1$	$1{,}700 \overset{\times}{\div} 1.2$	$1{,}500 \overset{\times}{\div} 1.1$
+	+	Heated(30 min. 56°C)	$19{,}700 \overset{\times}{\div} 1.1$	-	-
+	+	Dialyzed	-	-	$5{,}900 \overset{\times}{\div} 1.1$
		Mouse (NMS)			
+	+	Fresh	-	-	$5{,}600 \overset{\times}{\div} 1.1$

\# Results are expressed as geometric mean $\overset{\times}{\div}$ SE (n=5) of PFC per spleen assayed 4 days after iv injection of SRBC.

** Absorbed at 4°C 1x (Expt. 1) or 2x (Expt. 2) with mouse spleen (10^8 cells/0.5 ml of 1:2 dil. NHS) or with CTLL-A11 (murine cytotoxic T cell line; Expt. 3: 3.5×10^7 cells/0.5 ml of 1:2 dil. NHS).

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TABLE 4

EFFECT OF NORMAL MOUSE SERUM ON IMMUNE RESPONSE TO TNP-CONJUGATES IN SJL MICE

Injected i.v. on			Geom. Mean Anti-TNP PFC $\overset{\times}{\div}$ SE per Spleen in Response to
Day 0	Day 0	Day -1	TNP-Ficoll
Ag	Y-RCS		
	(2×10^7)	$(50\mu l)$	
+	-	-	43,652 $\overset{\times}{\div}$ 1.05
+	+	-	46,344 $\overset{\times}{\div}$ 1.05
+	+	Human Serum	93,110 $\overset{\times}{\div}$ 1.10

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TABLE 5

ENHANCEMENT BY HUMAN SERUM AND ISOLATED PLATELET RELEASATE OF ANTIBODY PRODUCTION IN SJL MICE[**]

Injected iv				
Day 0 SRBC (10[7])	Day 0 γ-RCS (2x10^{7})	Day -1 (50 µl)	Expt. 1 PFC/Spl	Expt. 2 PFC/Spl
+	-	-	$9,100 \overset{\times}{\div} 1.0$	$7,950 \overset{\times}{\div} 1.1$
+	+	-	$1,500 \overset{\times}{\div} 1.2$	$1,100 \overset{\times}{\div} 1.3$
+	+	NHS		
+	+	NHP		$4,000 \overset{\times}{\div} 1.5$
+	+	Platelet Lysate/Sup.	$27,550 \overset{\times}{\div} 1.1$	$18,600 \overset{\times}{\div} 1.1$
+	+	Thrombin[#]		$3,600 \overset{\times}{\div} 1.3$

* Human platelet releasate from thrombin incubated platelets (approx. 2×10^8/ml); 0.2 ml injected per mouse.

Amount of thrombin used was the same as that added to platelets to induce clumping (1 U/ml).

** Results are expressed as geometric mean $\overset{\times}{\div}$ SE (n=5) of PFC per spleen assayed 4 days after iv injection of SRBC.

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TABLE 6

ABROGATION OF CON A INDUCED SUPPRESSION OF THE ANTI-SRBC RESPONSE IN

CB6F$_1$·MICE BY A PLATELET DERIVED SERUM FACTOR [a]

Human Serum			Expt. 1	Expt. 2	Expt. 3	Expt. 4
Day 0 SRBC [b]	or Plt. Releasate	Day -1 Con A	PFC/Spleen	PFC/Spleen	PFC/Spleen	PFC/Splee
+	None	-	99,200 (1.1)	79,400 (1.2)	5,600 (1.1)	8,700 (1.1
+	None	50 µg	20,370 (1.1)[e]	-	-	-
+	Day -1[d]	50 µg	65,300 (1.0)[e]	-	-	-
+	None	5 µg	-	84,760 (1.2)[g]	1,540 (1.3)	4,400 (1.1
+	None	10 µg	-	29,660 (1.2)[f]	-	-
+	Day -1[c]	10 µg	-	63,250 (1.1)[f]	-	-
+	Day -1[c]	5 µg	-	120,980 (1.1)[g]	77,600 (1.2)	-
+	Day -1[d]	5 µg	-	-	44,900 (1.1)	51,590 (1.
+	Day 0[c]	5 µg	-	-	-	51,500 (1.

a) Results are expressed as geometric mean (∓ SE) [n=4] of PFC per spleen assayed 5 days after ip injection of SRBC.

b) SRBC were injected ip: 1 x 10^8 in Expt. 1; 5 x 10^7 in Expt. 2; 2 x 10^7 in Expts. 3 and 4.

c) 50 µl serum/mouse, i.v.

d) 0.1 ml 1:50 diluted platelet releasate (10^9 platelets/ml) injected i.v. 1-2 hrs before Con A p values are given for comparisons of values designated by same footnotes.

e) p<0.0001. f) p<0.01. g) p<0.05. h) p<0.0001

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TABLE 7

ABSORPTION OF IMMUNOREGULATORY FACTOR FROM SERUM BY CELLS FROM DIFFERENT LYMPHOID ORGANS

Day 0 SRBC	Day 0* Human Serum	Day -1 Con A	Day 5** PFC/Spleen Geom. Mean S.E.
+	None	-	$8,810 \times \div 1.1$ a)
+	Unabsorbed	-	$20,520 \times \div 1.2$ a) b)
+	None	+	$2,930 \times \div 1.1$
+	Unabsorbed	+	$14,300 \times \div 1.1$ b) c) d)
+	Absorbed 2 x 10^8 Spl	+	$3,100 \times \div 1.1$ c)
+	" 4 x 10^7 Spl	+	$6,870 \times \div 1.1$
+	" 4 x 10^7 LN	+	$5,570 \times \div 1.1$ d)
+	" 2 x 10^8 Thy	+	$13,870 \times \div 1.1$
+	" 4 x 10^7 Thy	+	$13,960 \times \div 1.1$

* One ml (1:2 diluted) serum was absorbed twice for 30 min. at 4^oC using half the number of CB6F$_1$ mouse cells indicated for each absorption.

Each CB6F$_1$ mouse received 0.2ml of 1:4 diluted unabsorbed or absorbed serum iv 1-2 hrs before 2 x 10^6 SRBC iv. Spl = spleen; LN = lymph node; Thy = thymus.

** p values are given for comparisons between values designated by the same footnote. n = 4.

a) p = .002.

b) N.S. (p=.081)

c) p<.0001.

d) p<.0001.

TABLE 8

ABSORPTION OF ACTIVITY BY HEPARIN-AGAROSE

Mice Injected With[a]		Geom. Mean PFC/Spleen on Day 5		
5 μg Con A	Additional Material	Expt. 1	Expt. 2	Expt. 3
–	None	9,330	15,850	7,700
+	None	4,020*	8,510	1,760
+	Rel.	11,450*	22,910	ND
+	Rel. absorbed with heparin-agarose	3,000*	7,410	4,210
+	Heparin-agarose eluate from Rel.	10,540*	26,060	11,940
+	Rel. absorbed with agarose	ND	ND	10,580

[a] Mice injected iv on day –1 with 5 μg Con A and on day 0 with 2×10^6 SRBC and platelet releasate (Rel.) or derivatives.

*Antilog of standard error = 1.2; for all other determinations, it is $\leq$ 1.1. (n=4). .

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TABLE 9

IMMUNOREGULATORY ACTIVITY OF PLATELET α GRANULE CONSTITUENTS

Mice Injected With[*]		Geom. Mean PFC/Spleen[+] on Day 5		
5 μg Con A	Additional Material	Expt. 1	Expt. 2	Expt. 3
-	None	16,090	12,450	32,590
+	None	8,000	4,880	12,920
+	Rel.	14,190	29,920	30,740
+	PF4[*]	ND	22,910	34,420
+	LA-PF4[**]	ND	9,440	ND
+	PDGF[≠]	8,370	ND	ND

[*] Mice injected iv on day -1 with 5 μg Con A and on day 0 with 2 x 10^6 SRBC plus

   releasate (Rel.) from 2 x 10^7 platelets or purified platelet proteins.

[**] 0.6 μg (Expt. 2) or 0.2 μg (Expt. 3) per mouse.

[≠] 0.003 μg per mouse.

[+] S.E. (antilog) ≤ 1.1; n = 4.

TABLE 10

REVERSAL OF IMMUNOREGULATORY ACTIVITY IN HUMAN SERUM BY GOAT ANTISERUM TO PF-4

Mice Injected With [*]		Geom. Mean PFC/Spleen [**]
5 µg Con A	Additional Material [≠]	on Day 5
-	None	8,720
+	None	4,380
+	Human Serum	51,590
+	Human Serum + goat anti-PF4	13,190
+	Human Serum + Nl goat serum	49,470
+	Goat anti-PF4	10,000

[**] S.E. (antilog) ≤ 1.1; n = 4.

[*] Mice injected with Con A and SRBC as in Table 4.

[≠] Human serum (0.05 ml) and/or goat antiserum to PF4 (0.008 ml) or normal goat serum (0.008 ml) were incubated for 20 min. at $4^{\circ}C$ prior to injection.

TABLE 11

EFFECT OF VARIOUS SERA WITH OR WITHOUT PRIOR ABSORPTION
WITH SPLEEN CELLS ON SUPPRESSION BY CON A OF RESPONSE TO SRBC
IN VIVO*

Injections** on Day −1	% of Control Response (PFC/Spleen)
None	100
	(9,600 − 27,900)
Con A	50
Con A + Mouse Serum	234
Con A + Absorbed NMS	44
Con A + Human Serum	144
Con A + Adsorbed HS	67
Con A + Fetal calf serum	183
Con A + Absorbed FCS	62

*Con A (8 µg) was injected iv on Day −1; 2×10^6 SRBC were injected iv on day 0 into $(CB6)F_1$ mice. PFC/spleen were determined on day 4. Each group consisted of 5 mice. Results of 3 experiments were combined. Range of control PFC/spleen is given in parentheses.

**Serum was injected iv 1 hour prior to Con A as 0.2 ml of 1/4 dilution. Sera were absorbed twice, each time using 5×10^8 spleen cells per ml of 1:2 diluted serum for 30 min. at 4°C.

TABLE 12

LACK OF EFFECT OF PLATELET RELEASATE ON SUPPRESSION BY CON A OF THE IMMUNE
RESPONSE OF LYMPH NODE CELLS TO TNP-B.abortus* IN VITRO

Additions to Culture		% of Control Response (anti-TNP PFC/dish)$^\theta$	
Platelet Releasate**	Con A$^{\neq}$	Expt. 1	Expt. 2
−	−	100	100
		(438)	(720)
+	−	115.3	118.1
−	High	14.4	11.8
+	High	26.3	14.6
−	Low	25.8	29.2
+	Low	33.8	16.8

*TNP-BA 100 μg and 5×10^6 lymph node cells per culture.

**Platelet releasate added per culture contained 0.15μg (Expt. 1) or 0.22μg

(Expt. 2) of human PF_4.

$^{\neq}$Con A added was 8 μg (Expt. 1) or 4 μg (Expt. 2) per culture for high doses

and 4 μg (Expt. 1) and 2 μg (Expt. 2) for low doses.

$^\theta$Determined on day 4 of culture; values represent averages of duplicates not

differing by more than 10%. PFC/dish in control cultures are given in

parentheses.

TABLE 13

ADHERENCE OF CON A INDUCED SUPPRESSOR CELLS TO PF4-COATED BEADS OR DISHES

SPLEEN CELLS ADDED TO DISHES	% of Control Response (PFC/dish)	
	Series 1*	Series 2**
Normal mouse: 4×10^6 unfractionated	100 (227-305)	100 (840-933)
Con A injected mouse: 4×10^6 unfractionated	57.0± 5.2	64.1± 3.7
Con A injected mouse: 4×10^6 PF4 non-adherent	103.3±31.9	138.6±30.9
Con A injected mouse: 4×10^6 control non-adherent	78.6±33.7	73.2±13.0
Con A injected mouse: PF4 adherent	33.5± 6.6	45.3±11.9
Con A injected mouse: control adherent	70.9±16.6	75.7±21.6

*Series 1 experiments (n=3) were done with 10^7 target normal BALB/c spleen cells and 50 µl 0.5% SRBC per dish. Adherence was performed by rotating spleen cells from Con A-injected mice with heparin-agarose beads coated with PF4 or with uncoated beads for 1 hour at 4°C. The beads were gently but extensively washed. Then adherent cells were vigorously triturated off the beads and used at 0.3×10^6 per dish.

**Series 2 experiment (n=3) were done with $1.5-2 \times 10^7$ target spleen cells per dish. Adherence was performed for 1 hour at 37°C on dishes coated with PF4 from mouse serum or on control dishes coated with diluent. Only readily obtained cells were used as nonadherent, while even loosely adherent cells were considered adherent. Adherent cells used at $1-4 \times 10^6$ per dish. Values in parentheses represent ranges of control PFC/dish in the three experiments in each series.

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TABLE 14

EFFECT OF LECTINS AND LYMPHOKINES ON IMMUNE RESPONSE OF BALB/c MICE TO

TYPE 14 PNEUMOCOCCAL POLYSACCHARIDES

Antigen Dose (Day 0)	Additional Injections	PFC/Spleen * (Day 4-5)	Serum Agglutinin Titers (Log$_2$ 1/Titer ± SE)	
			Day 4-5 *	Day 7 **
0.5 µg	None	389 $\overset{\times}{\div}$1.9	4.4 ± 0.7	6.1 ± 0.3 (≤ 2.0)
0.5 µg	Con A + PHA (Day 2)	146 $\overset{\times}{\div}$1.7	6.0 ± 0.4	6.8 ± 0.8 (3.5 ± 0.7)
25 µg	None	598 $\overset{\times}{\div}$1.1	8.3 ± 0.3 (≤ 2.0)	7.8 ± 0.3 (≤ 2.0)
25 µg	Con A + PHA (Day 2)	8,678 $\overset{\times}{\div}$1.5	9.0 ± 0.7	8.4 ± 0.4 (4.3 ± 0.9)
25 µg	None	663 $\overset{\times}{\div}$1.3 (12)	4.5 ± 0.5 (12)	6.1 ± 0.2 (9) (5.2 ± 0.3)
25 µg	Con A + PHA (Day 2)	1,807 $\overset{\times}{\div}$1.2 (7)	5.7 ± 0.2 (7)	7.1 ± 0.4 (9) (7.4 ± 0.5)
25 µg	Lymphokines (Days 1 + 2)	1,277 $\overset{\times}{\div}$1.4 (8)	6.1 ± 0.6 (8)	7.8 ± 0.3 (6) (8.3 ± 0.3)
25 µg	8-Mercaptoguanosine (Day 0)	2,599 $\overset{\times}{\div}$1.3 (4)	5.8 ± 0.3 (4)	7.0 ± 0.6 (5) (6.1 ± 0.8)
Killed pneumococci 10^9	None	180,000 (1)	13 (1) (8.5)	14 (1) (7)
10^8	None	12,000 $\overset{\times}{\div}$1.04 (2)	8.8 (2)	8.4 ± 0.5 (6) (6.8 ± 0.5)

pt. 1, Day 5; Expt. 2, Day 4.

rum titers after incubation with 0.1M 2-mercaptoethanol are given in parentheses.

TABLE 15

EFFECT OF LECTINS INJECTED 2 DAYS AFTER ANTIGEN ON THE IMMUNE RESPONSE TO

PNEUMOCOCCAL POLYSACCHARIDE TYPE 3

Immunogen 0.5 μg, ip	Adjuvants (Day 2)	Log_2 1/Serum Antibody Titers On[**]			
		Day 5		Day 10	
		19S	7S	19S	7S
pn. ps. type 3 – MTP	None	7.4 ± 0.1	2.0	6.7 ± 0.3	2.0
pn. ps. type 3	None	4.0 ± 1.1	2.0	3.6 ± 1.0	2.0
pn. ps type 3 – MTP	Lectins[*]	8.6 ± 0.2	2.0	6.2 ± 3.0	2.5 ± 0.2
pn. ps. type 3	Lectins[*]	4.3 ± 0.8	2.0	4.9 ± 0.3	2.5 ± 0.3

[*] Con A 75 μg ip and PHA 20 μg iv.

[**] Log_2 ± SE (n = 5) of 1/agglutinin titer against pn. ps. type 3 coated sheep R determined with (7S) or without (19S) prior incubation for 1 hour at room temperature in 0. 2-mercaptoethanol.

TABLE 16

EFFECT OF ANTIGEN DOSE ON IMMUNE RESPONSE OF BALB/c MICE TO PNEUMOCOCCAL POLYSACHARIDE TYPE 14

Log_2 1/titer of Hemagglutinin ± SE

Mice[#]	Dose of Antigen (n)	Primary Response*			Secondary Response**	
		Day 5 19S	Day 10 19S 7S	Day 15 19S 7S	Day 20 19S 7S	Day 25 19S 7S
Yg	25µg (30)	6.8±0.2	5.2±0.2 4.0±0.3	4.6±0.3 3.1±0.3	8.0±0.3 4.7±0.3	6.9±0.3 4.8±0.4
Yg	5µg (19)	5.2±0.2	3.8±0.3 2.7±0.2	4.7±0.6 2.9±0.5	7.5±0.5 4.4±0.4	6.1±0.4 4.5±0.4
Yg	0.5µg (9)	4.7±0.5	4.6±0.3 3.0±0.6	3.7±0.5 3.0±0.5	4.8±0.1 <2.5	6.9±0.3 2.5±0.3
Yg	0.2µg (13)	2.8±0.3	2.8±0.3 <2.5	2.4±0.4 <2.5	6.2±0.6 3.3±0.4	4.9±0.5 3.3±0.4
Yg	0.05µg (4)	<2.5	3.4±0.2 2.5±0.3	ND	6.6±0.2 4.5±0.7	5.0±0.3
Yg	0.01µg (7)	<2.5	3.0±0.3 2.9±0.3	ND	7.0±0.4 4.3±0.5	2.8±0.2 <2.5
Yg	0 (34)	<2.5	<2.5	ND	7.7±0.2 3.5±0.2	5.4±0.3 3.3±0.2
Aged	25µg (5)	6.4±0.5	6.3±0.6 3.4±0.8	ND	9.8±0.2 5.9±0.3	ND
Yg NuNu	25µg (19)	6.5±0.5	4.8±0.3 3.0±0.3	4.3±0.4 <2.5	6.9±0.4 3.4±0.3	6.3±0.5 2.5±0.2

*Primary injection of pneumococcal polysaccharide type 14 was ip on day 0.

**Secondary injection of 5 x 10^8 killed penumococci type 14 was ip on day 15.

[#] Young mice were 6-10 wks of age; aged mice were 21 months old.

TABLE 17

EFFECT OF ANTIGEN DOSE ON IMMUNE RESPONSE OF BALB/c MICE TO
PNEUMOCOCCAL POLYSACCHARIDE TYPE 3

Log_2 1/titer of Hemagglutinin ± SE

Dose of Antigen (n)	Primary Response*					Secondary Response**			
	Day 5 19S	Day 10 19S	7S	Day 15 19S	7S	Day 20 19S	7S	Day 25 19S	7S
5–10µg (22)	<25	2.6±0.2	<2.5	3.0±0.3	<2.5	3.0±0.2	<2.5	3.5±0.1	<2.5
0.1µg (33)	8.6±0.2	6.7±0.2	2.9±0.3	4.8±0.2	3.0±0.4	6.3±0.2	3.8±0.3	5.4±0.2	<2.5
"	"	"		"		≠6.6±0.2	4.0±0.3	5.8±0.3	2.5±0.2
0.005µg (17)	2.9±0.3	2.6±0.2	<2.5	<2.5		≠5.5±0.5	<2.5	5.2±0.5	<2.5
0.002µg (5)	<2.5	<2.5		<2.5		4.0±0.3	<2.5	5.8±0.2	<2.5
0 (12)	<2.5	<2.5		<2.5		5.8±0.5		4.8±0.3	<2.5

*Primary injection of pneumococcal polysaccharide type 3 was ip on day 0.

**Secondary injection of 1 X 10^8 killed pneumococci was ip on day 15.

≠Secondary injection was 0.1 µg pps 3 on day 15 (in 10/33 mice in the 0.1 µg group and in all the mice of the 0.005 µg group).

TABLE 18

TOLERANCE INDUCTION BY HIGH DOSES OF PNEUMOCOCCAL
POLYSACCHARIDE TYPE 3 IN Nu/Nu BALB/c AND (CB6) F1 MICE

Log_2 1/titer of Hemagglutinin ± SE

Mouse Strain	n	Primary Dose of Antigen	*Primary Response		**Secondary Response
			Day 5 19S	Day 10 19S	Day 15 19S
Nu/Nu BALB/c	5	5µg	<2.5	<2.5	<2.5
	14	0.1µg	4.6±0.3	5.4±0.7	3.0±0.6
	2	None	<2.5	<2.5	6.3±0.1
(CB6)F1	9	5µg	<2.5	2.8±0.3	<2.5
	4	5µg≠	<2.5	5.5±0.5	6.5±0.4
	3	0.1µg	9.0±0.4	5.3±0.4	6.8±0.1
	5	None	<2.5	<2.5	6.6±0.4
	3	$1.2x10^9$ killed pneumococci	<2.5	<2.5	<2.5
	3	$1x10^8$ killed penumococci	5.2±0.3	5.8±0.3	6.5±0.5

*Primary injection of pneumococcal polysaccharide type 3 or killed pneumococci type 3 was ip on day 0.

**Secondary injection of $1X10^8$ killed pneumococci type 3 was ip on day 10.

≠10 µg detoxified endotoxin was injected ip on day 4.

TABLE 19

INDUCTION OF LOW DOSE TOLERANCE TO PNEUMOCOCCAL POLYSACCHARIDE TYPES 3 AND 14

Polysaccharide[*] Type	n	Dose of pps on Day-3	Day 0	Log_2 1/titer of Hemagglutinin ± SE		
				Day 5 19S	Day 10 19S 7S	Day 15 19S 7S
pps 14	5	0.2µg	25µg	3.8±0.1[∅]	3.2±0.4[≠] <2.5	3.5±0.4 <2.5
	30	None	25µg	6.8±0.2	5.2±0.2 4.0±0.3	4.6±0.3 <2.5
pps 3	11	0.002µg	0.1µg	4.1±0.7[≠]	3.5±0.4[≠] <2.5	3.5±0.4 <2.5
	11	0.004µg	0.1µg	3.0±0.4[∅]	3.1±0.3[∅] <2.5	3.1±0.3[o] <2.5
	27	0.005µg	0.1µg	3.1±0.4[∅]	3.4±0.3[∅] <2.5	3.1±0.4[o] <2.5
	33	None	0.1µg	8.6±0.2	6.7±0.2 2.9±0.3	4.8±0.2 3.0±0.4

*Pneumococcal polysaccharides were injected ip.

[∅] p vs. control <.0001

[≠] p vs. control <.001

[o] p vs. control <.01

TABLE 20

LACK OF LOW DOSE TOLERANCE TO PNEUMOCOCCAL POLYSACCHARIDE TYPE 3
IN AGED AND ATHYMIC BALB/c MICE

$Log_2 1$/titer of Hemagglutinin ± SE

| Mice | Dose of pps on | | Day 5 | Day 10 | | |
	Day-3	Day 0	19S	19S	7S	(n)
Aged	0.005µg	0.1µg	5.9±0.7	7.1±02	4.4±0.7	(8)
	None	0.1µg	6.7±0.9	6.7±0.8	4.2±0.6	(5)
Nu/Nu	0.005µg	0.1µg	4.8±0.6	5.3±0.5	2.6±0.4	(9)
	None	0.1µg	4.6±0.3	5.4±0.7	3.4±0.4	(14)

*Aged=18 months

TABLE 21

EFFECT OF ENDOTOXIN ON IMMUNE RESPONSES TO PNEUMOCOCCAL
POLYSACCHARIDE TYPE 14

Log_2 1/titer of Hemagglutinin ± SE

Mouse Strain	n	D-LPS* Dose	D-LPS* Day	** Primary Response Day 5	Day 10 19S	Day 10 7S	Day 15 19S	Day 15 7S	***Secondary Response Day 20 19S	Day 20 7S	Day 25 19S	Day 25 7S
BALB/c	5	50µg	0	6.3±0.2	5.5±0.2	4.9±0.2	5.3±0.2	3.7±0.6	ND		ND	
	9	10µg	2	7.1±0.3	6.0±0.3	4.9±0.6	5.0±0.3	3.8±0.4	8.9±0.4	5.4±0.4	8.2±0.2	6.9±0.3
	12	10µg	3	7.9±0.4	6.5±0.4≠	5.6±0.5	6.3±0.4≠≠	5.8±0.6	8.7±0.2	5.4±0.4	8.5±0.3≠≠	6.8±0.3
	8	10µg	4	8.1±0.4	6.3±0.5	4.8±0.7	6.3±0.6	5.3±0.7	9.1±0.2	5.8±0.5	8.4±0.5	6.3±0.7
	30	–	–	6.8±0.2	5.2±0.2	4.0±0.3	4.6±0.3	3.1±0.3	8.0±0.3	4.7±0.3	6.9±0.3	4.8±0.4
BALB/c u/Nu	21	10µg	3–4	5.8±0.3	4.6±0.3	3.0±0.2	ND		6.9±0.2	3±0.2	6.4±0.2	<2.5
	14	–	–	6.5±0.5	4.8±0.3	3.0±0.3	3.9±0.5	<2.5	6.5±0.3	3.3±0.3	5.9±0.3	2.6±0.2

D LPS=Detoxified endotoxin from S. typhimurium.

*Primary injection of pneumococcal polysacharide Type 14 25 µg was ip on day 0.

**Secondary injection of 5×10^8 killed pneumococci type 14 was ip on day 15.

p vs. control = .05

≠ p vs. control = .01

TABLE 22

EFFECT OF ENDOTOXIN ON LOW DOSE TOLERANCE TO PNEUMOCOCCAL POLYSACCHARIDES

Polysaccharide* Type	n	Dose of pps Day-3	Day 0	Day≠ of D-LPS Injection	Log$_2$ (1/titer) ± SE Day 5 19S	Day 10 19S	7S	Day 15 19S	7S
pps-14	5	0.2µg	25µg	0	5.5±0.4ᵩ	4.9±0.5	4.0±0.7	3.8±0.5	2.9±0.5
	5	0.2µg	25µg	-3	5.5±0.4	4.9±0.3	4.3±0.6	3.6±0.6	3.1±0.4
	5	0.2µg	25µg	—	3.8±0.1ᵩ	3.2±0.4	<2.5	3.5±0.2	<2.5
	30	None	25µg	—	6.8±0.2	5.2±0.2	4.0±0.3	4.6±0.3	3.1±0.3
pps-3	3	0.002µg	0.1µg	0	4.5±0.5	5.7±0.6		6.7±0.1	
	3	0.002µg	0.1µg	—	<2.5	<2.5		<2.5	
	33	None	0.1µg	—	8.6±0.2	6.7±0.2		4.8<0.2	

*Pneumococcal polysaccharides injected ip.

≠D-LPS = detoxified endotoxin injected ip. (50 µg).

ᵩDifference between these two values: p<.05.

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TABLE 23

EFFECT OF 8-MERCAPTOGUANOSINE (8-MG) ON IMMUNE
RESPONSE TO PNEUMOCOCCAL POLYSACCHARIDE TYPE 14*

Log_2 1/titer of Hemagglutinin ± SE

| Dose of | Day 7 | | Day 14 | | |
8-mg	19S	7S	19S	7S	(n)
10 mg ays 1,2,3	5.2±1.0	4.4±0.7	3.4±0.6	3.3±0.4	(5)
30 mg Day 0	7.0±0.5	5.9±0.6	7.0±0.5	6.3±0.5	(5)
None	5.6±0.3	3.8±0.3	4.7±0.4	3.2±0.4	(17)

25 µg pneumococcal polysaccharide type 14 injected ip on day 0.

TABLE 24

IMMUNE RESPONSE TO PNEUMOCOCCAL POLYSACCHARIDE
EMULSIONS WITH AND WITHOUT MURAMYL DIPEPTIDE

Log_2 1/titer of Hemagglutinin ± SE

Antigen on Day 0	*Adjuvant Mixture	Day 7 19S	7S	Day 14 19S	7S	Day 21 19S	7S
pps–14 25μg,ip	Sq/Arl + Nor–MDP	6.8±0.8	5.5±1.2	6.3±0.5	6.0±0.5	5.2±1.1	3.7±1.4
	Sq/Arl	6.0±0	4.2±0.4	5.7±0.3	4.8±0.4	5.0±0	3.7±0.3
	Pluronic/Arl+ T–MDP	5.5±0	2.5±0.4	5.7±0.3	5.8±0.3	4.5±0.2	2.5±0.2
	Pluronic/Arl	6.2±0.1	2.8±0.7	6.3±0.3	4.7±0.3	5.7±0.5	<2.5
	Saline	5.6±0.3	3.8±0.3	4.7±0.4	3.2±0.4	5.5±0.4	2.5±0.3
pps–3 0.1μg,ip	Sq/Arl+ Nor–MDP	7.9±0.2	4.4±0.5	5.8±0.1	<2.5	5.5±0.3	<2.5
	Sq/Arl	7.1±0.1	<2.5	6.0±0.2	<2.5	5.3±0.3	3.0±0.5
	Pluronic/Arl+ T–MDP	4.3±0.7	<2.5	4.3±0.5	<2.5	4.3±0.3	<2.5
	Pluronic/Arl	3.2±0.1	<2.5	3.0±0.1	<2.5	3.0±0.5	<2.5
	Saline	7.3±0.3	2.8±0.2	5.4±0.4	<2.5	5.1±0.4	<2.5

*Adjuvant – Nor–MDP   100μg/mouse

Threonyl–MDP (T–MDP)   20 μg/mouse

Squalene/Arlacel (Sg/Arl) 0.1 ml/mouse

Pluronic/Arlacel (Pluronic,. ⁻ ⁻ 1 ml/mouse

n=3; except control group where n=17 for pps 14 and n=8 for pps 3.

G. J. Thorbecke, M.D.

TABLE 25

EFFECT OF PF4 RELEASED FROM PLATELETS IN THE PRESENCE OR

BSENCE OF APMSF ON LOW DOSE TOLERANCE TO PNEUMOCOCCAL POLYSACCHARIDE TYPE 3

Antigen Dose* erizing y -3	Immunizing Day 0	PF4**	APMSF-** PF4	Log_2 1/titer ± SE Day 5	Day 10	n
g)	(µg)					
e	0.1	—	—	7.0 ± 0.3≠	5.8 ± 0.3≠	10
05	0.1	—	—	2.9 ± 0.6	3.9 ± 0.3	5
05	0.1	Day -3	—	4.5 ± 0.3≠	5.1 ± 0.3≠	5
05	0.1	Day 0	—	3.2 ± 0.3	3.8 ± 0.3	5
05	0.1	—	Day -3	2.5 ± 0.4	4.0 ± 0.2	5
05	0.1	—	Day 0	3.2 ± 0.5	3.4 ± 0.4	5

Antigen injected ip.

0.5µg injected iv.

ignificantly different from group receiving 0.005 µg followed 3 days

:er with 0.1 µg (line 2).

35
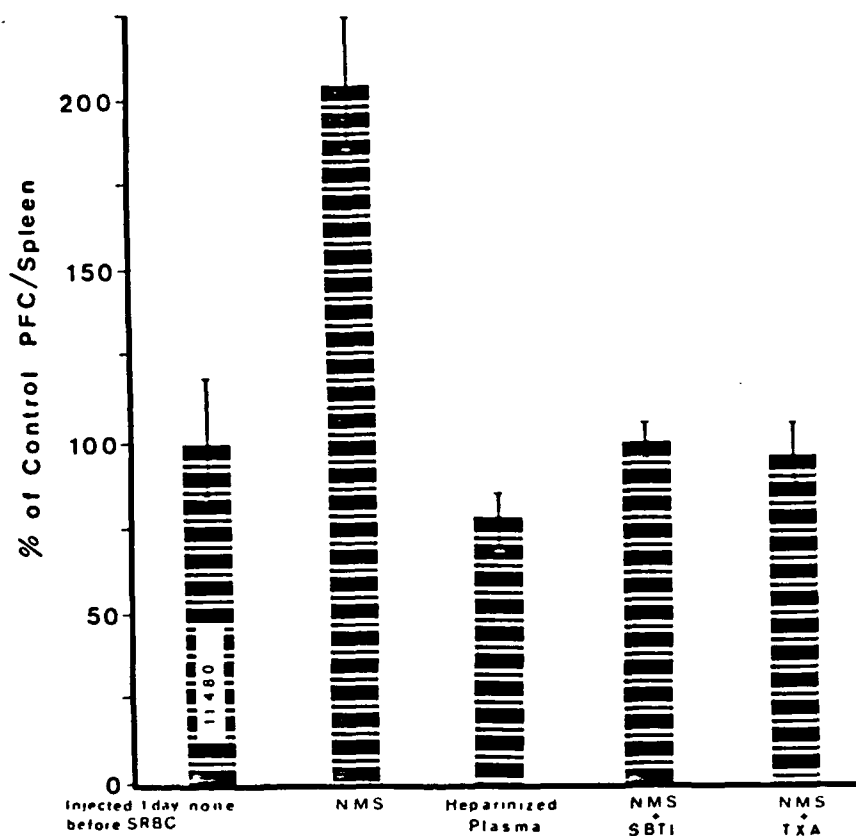
Legend for Figures:

Fig. 1. Effect of proteolytic enzyme inhibitors on appearance of antibody enhancing factor in serum during clotting of blood:

Splenic PFC response 4 days after iv injection of 10^7 SRBC (without γ-RCS) in SJL mice, who were also injected iv, 1 day before antigen, with 0.05 ml of normal mouse serum (NMS) which had been produced by clotting of whole blood in the presence of tranexamic acid (TXA, 50 μM) or of soybean trypsin inhibitor (SBTI, 10 μg/ml), or with plasma produced from heparinized blood. Results are expressed as °/° ($\overset{X}{\cdot}$ SE) of control (n = 5) and the total PFC per spleen in the control group is indicated in the bar.
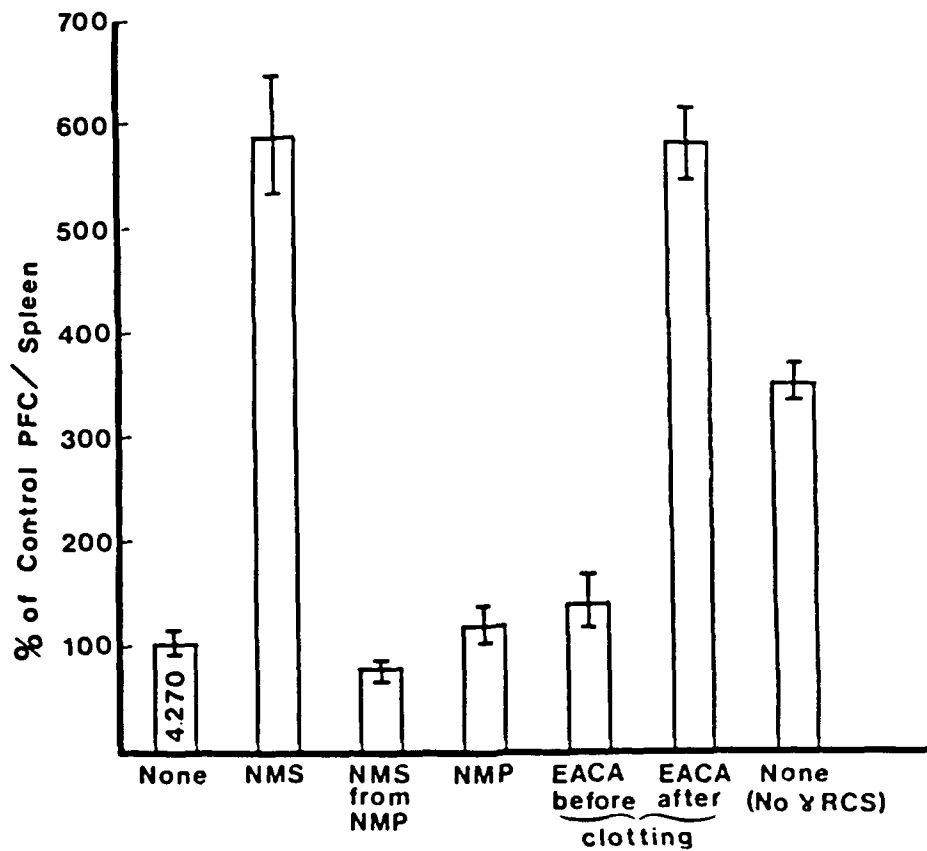
Fig. 2. Clotting in the presence of cells is needed for appearance of antibody enhancing factor in serum:

Splenic PFC response 4 days after iv injection of 10^7 SRBC and 2 x 10^7 γ-RCS in SJL mice, who were also injected iv, 1 day before antigen, with 0.05 ml of normal mouse serum (NMS), normal mouse plasma (NMP), NMS prepared after centrifugation of citrated blood by addition of Ca^{++} (NMS from NMP), or NMS to which EACA (25 μM) was added either before or after clotting. The response of mice injected with 10^7 SRBC without γ-RCS is also represented (None, no γ-RCS). Results are expressed as °/° ($\overset{x}{.}$ SE) of control (n = 4-5) and the total PFC per spleen in the control group is indicated in the bar.
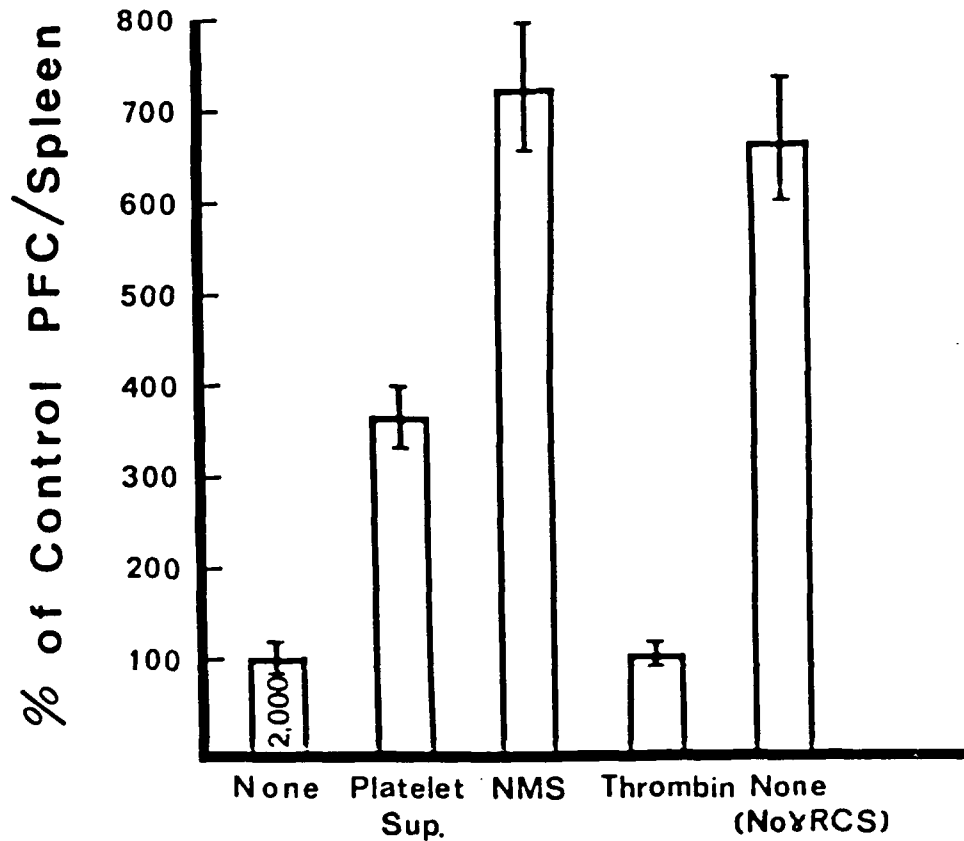
Fig. 3. Effect of platelet product on the immune response:

Splenic PFC response 4 days after iv injection of 10^7 SRBC and 2 x 10^7 γ-RCS in SJL mice, also injected iv, 1 day before antigen, with 0.05 ml of thrombin containing clotted platelet supernate, normal mouse serum (NMS) or thrombin alone. Results are expressed as °/° ($\overset{X}{\sim}$ SE) of con trol (n = 5) and the total PFC per spleen in the control group is indicated in the bar.
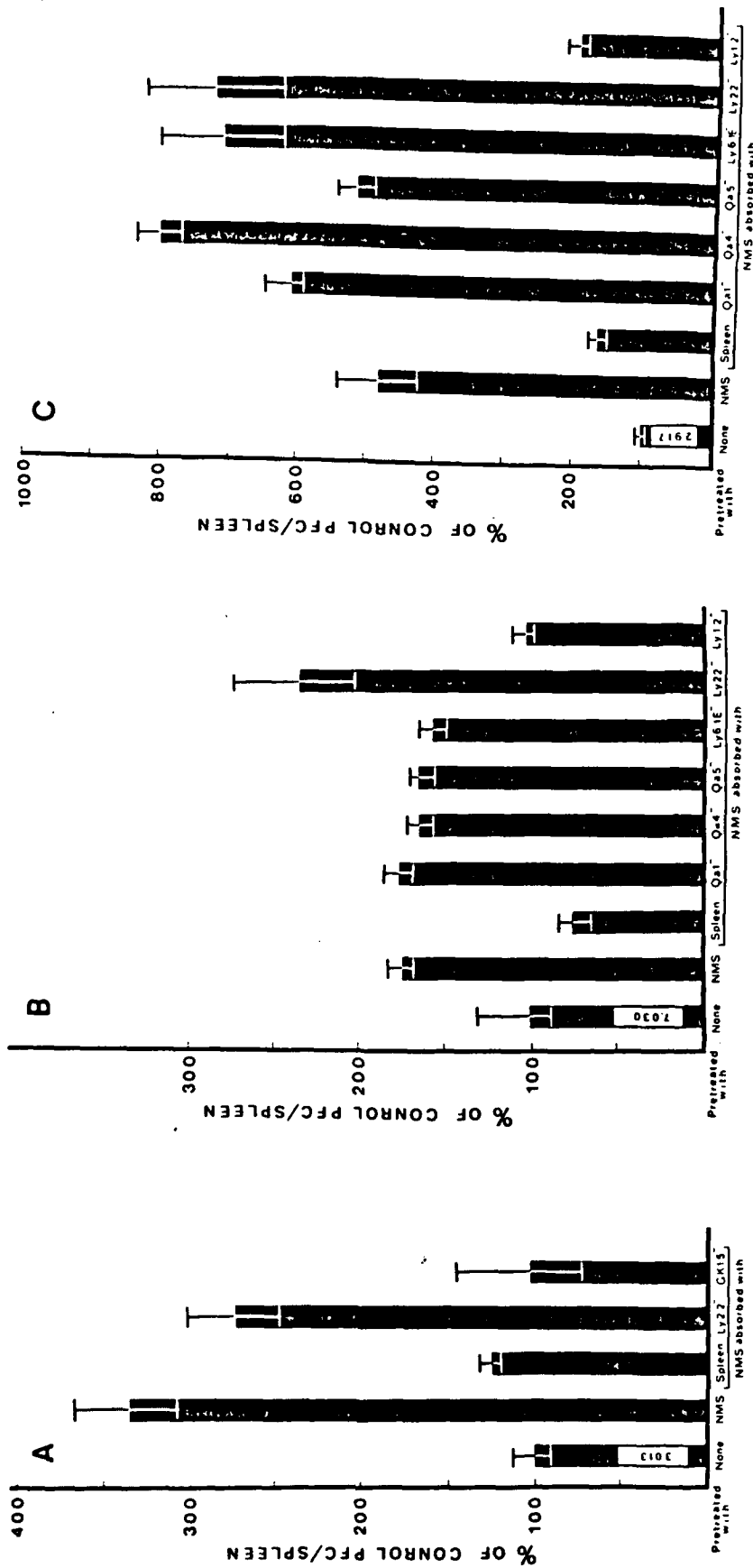
Identification of the subset and phenotype of cells able to absorb the immunoregulatory serum factor from serum.

Splenic PFC responses 5 days after simultaneous iv injection of 10^7 SRBC and 2×10^7 γ-RCS in SJL mice. Some groups of mice were also injected iv with 0.05 ml of unabsorbed normal mouse serum or serum absorbed (2X, see legend Table 3) with whole spleen cell suspension or with spleen cell suspension from which different subpopulations of cells were eliminated by treatment with mAbs. and complement (indicated as negative for the subpopulation which was killed). The response of mice injected with 10^7 SRBC without γ-RCS is represented as "None". Results are expressed as percent (± SE) of control (n=4) and the total PFC per spleen in control groups is indicated in the relevant bars for each of the three experiments. The responses are high in those groups of mice where the spleen cell suspension left after treatment with mAb and C was unable to absorb the augmenting factor from the serum.
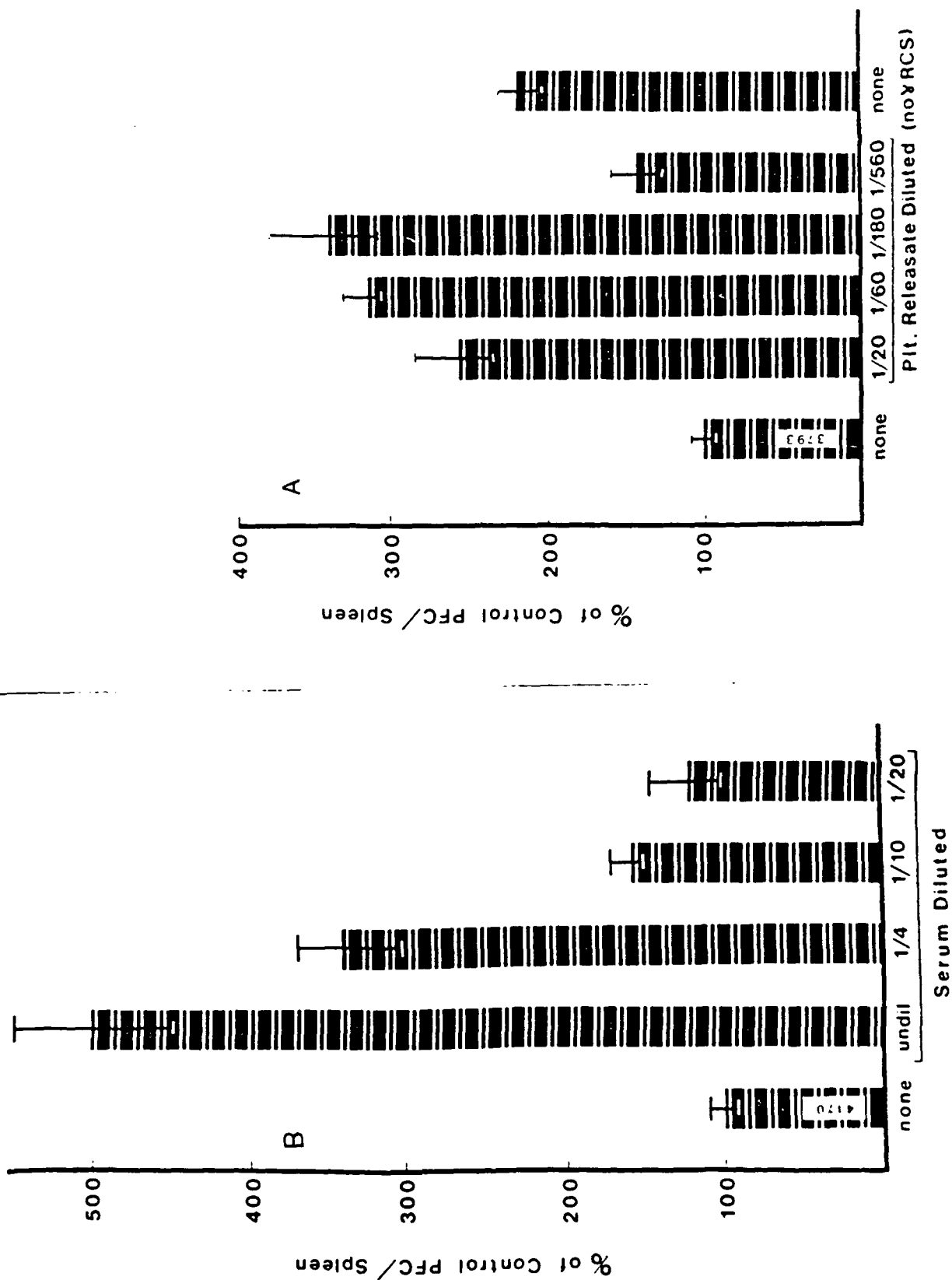
Fig. 4

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Fig. 5 -- Splenic PFC produced in response to injection of suppressive agent (γ-RCS), SRBC, and different concentrations of (A) human serum and (B) releasate from 10^9 platelets per ml. The geometric mean for PFC per spleen in the control suppressed mice is indicated in the first bars.
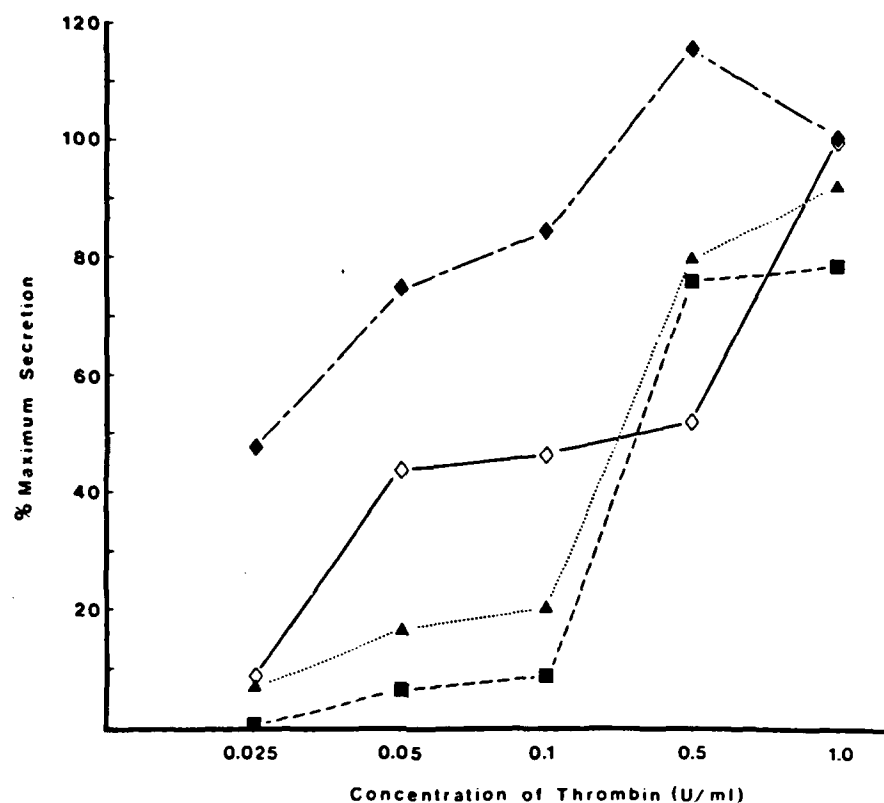
40

Fig.6-- Secretion of PF4 (◆), IA (◇) ^{14}C-serotonin (▲), and
β-glucuronidase (■) from platelets stimulated with thrombin.
The geometric mean for PFC per spleen was 4126 in mice injected
with suppressive agent (γ-RCS), SRBC and releasate made with 1 U/ml
thrombin, and was 339 in mice given γ-RCS and SRBC and no releasate.
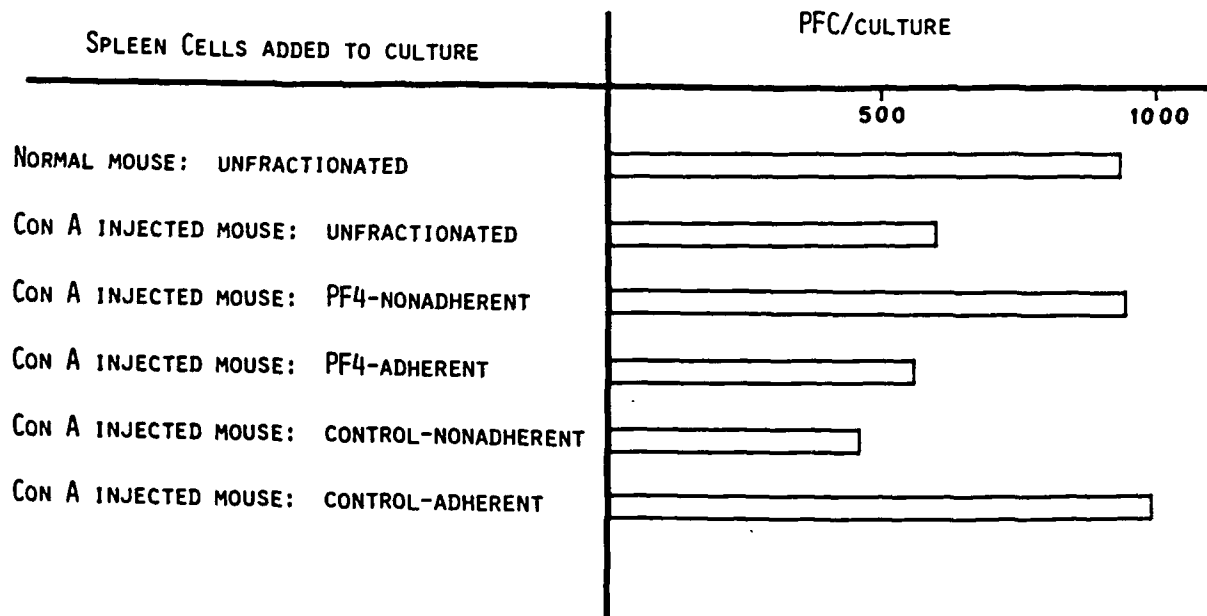
PF4 - ADHERENCE OF CON A INDUCED SUPPRESSOR CELLS

Fig. 7: Adherence of Con A-induced suppressor cells to mouse PF4-coated dishes. Responding cells were normal BALB/c spleen cells, 2×10^7 per cultures, to which were added 4×10^6 spleen cells either from an untreated normal or from a con A injected BALB/c mouse (1 day before culture, 30 µg per mouse iv). The spleen cells from the Con A injected mouse were also fractionated by adherence for 1 hr at 37°C to mouse serum PF4 coated 60 mm dishes (20 µg/dish, 2 hrs at room temp.). Non-adherent cells were added at 4×10^6/dish, adherent cells at 2×10^6/dish. Control-fractionation was in dishes coated with PF4-free plasma protein containing diluent. The same diluent was used for blocking the PF4-coated dishes. All cultures received 50 µl .5% SRBC on day 0 and PFC per dish were determined on day 4.
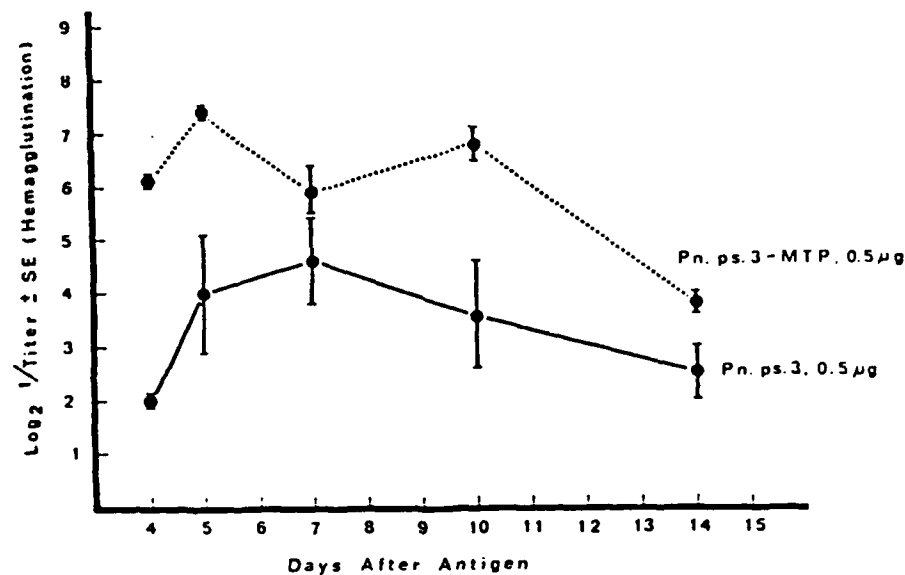
Fig. 8: Enhanced immunogenicity of ip injected MTP-conjugate of pneumococcal polysaccharide type 3 (pn. ps. 3) as compared to unconjugated pn. ps. 3. Serum agglutinin titers on pn. ps. 3 coated sheep RBC were determined by doubling dilutions after absorption with unconjugated sheep RBC (n = 3-7; data represent a composite of 3 experiments). Sera were also examined after treatment with 0.1M 2-mercaptoethanol, but no 7S antibody was found. A recent repeat of these experiments shows that simultaneous injection of 0.5 μg pn. ps. 3 and various MDP preparations, MDP, NOR-MDP and murabutide, each tested at 50 and 200 μg, induces much lower responses than does 0.5 μg of the pn. ps 3-MTP conjugate.

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SPECIFICITY OF LOW DOSE TOLERANCE TO PPS 3 AND 14

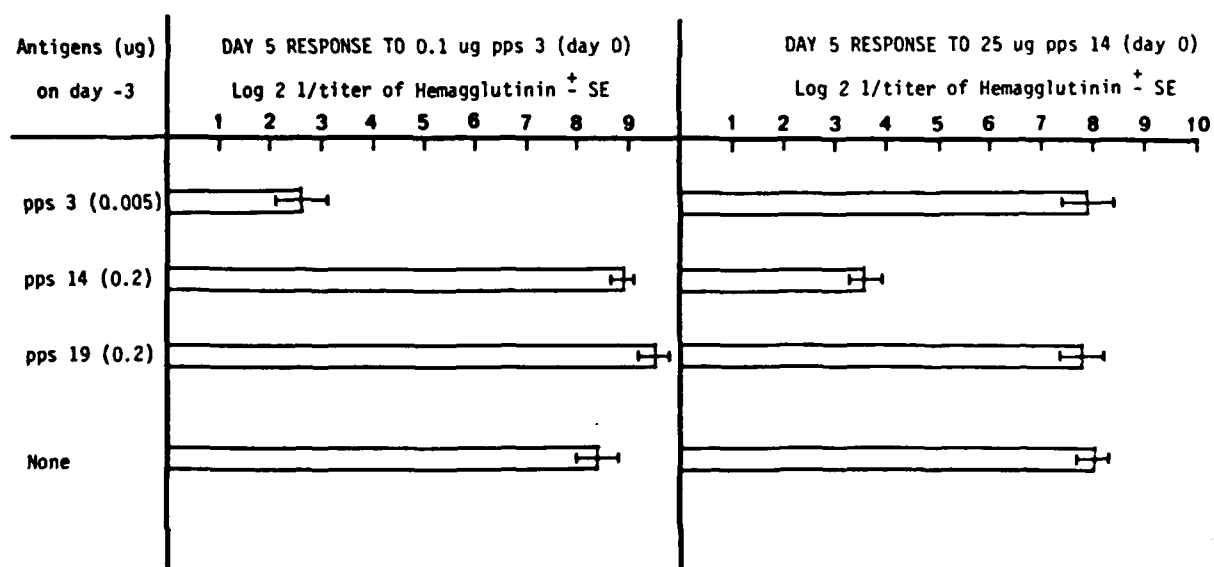| Antigens (ug) on day -3	DAY 5 RESPONSE TO 0.1 ug pps 3 (day 0) Log 2 1/titer of Hemagglutinin $\pm$ SE	DAY 5 RESPONSE TO 25 ug pps 14 (day 0) Log 2 1/titer of Hemagglutinin $\pm$ SE
pps 3 (0.005)	~2.5	~7.5
pps 14 (0.2)	~8.5	~4
pps 19 (0.2)	~8.5	~7.5
None	~8	~8

Fig. 9: Specificity of low dose tolerance to pps 3 and 14. Suboptimal antigen doses were injected ip on day −3 followed by optimal immunogenic doses of either pps 3 (0.1 µg) or of pps 14 (25 µg) on day 0. The 19S serum antibody titers (log$_2$ of 1/titer ± SE) for day 5 are represented (n=5).
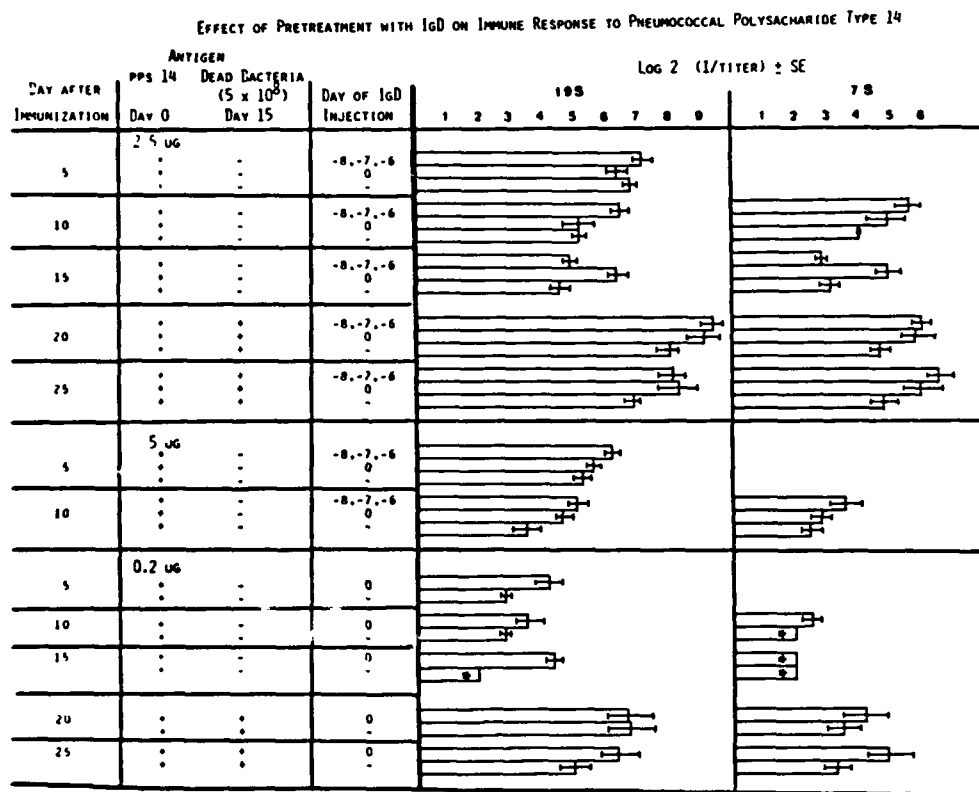
44

Fig. 10: Effect of IgD on immune response to pps 14. Groups of mice received different doses of pps 14 on day 0 either simultaneously with IgD (n= 9) or 1 week after pretreatment with 3 daily ip injections of IgD (n = 5 to 13). Mice received approx. 1.5 mg of TEPC 1017 IgD per injection. $\log_2$ of reciprocals of passive hemagglutinin titers determined without (19S) and with incubation in 0.1 M 2-mercaptoethanol (7S). Values indicated with * represent $\log_2$ 1/titer of <2.5.

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EFFECT OF HUMAN PLATELET RELEASATE (PR) ON IMMUNE RESPONSE TO PNEUMOCOCCAL POLYSACCHARIDE TYPE 14
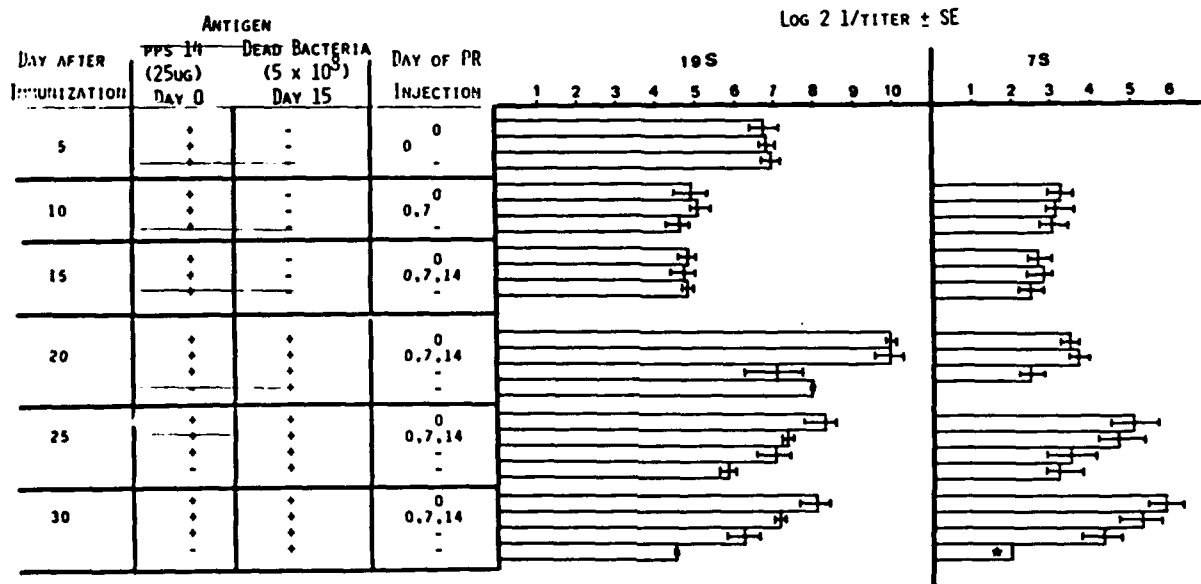
Fig. 11: Effect of platelet releasate on primary and secondary responses of BALB/c mice to an optimal dose of pps 14. Primary injection (day 0) was 25 µg pps 14 ip, secondary injection was 5×10^8 killed pneumococci type 14 ip. Platelet releasate was injected iv as equivalent of releasate from 2×10^7 human platelets per mouse (approx. 0.2–0.5 µg PF4 per mouse, either on day 0 only or at weekly intervals).

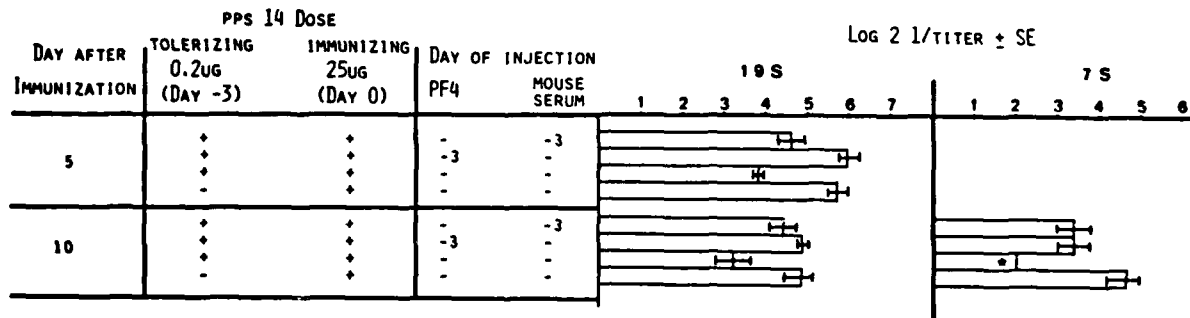EFFECT OF PLATELET RELEASATE ON IMMUNE RESPONSE TO PNEUMOCOCCAL POLYSACHARIDE TYPE 14

Fig. 12: Prevention of low dose tolerance in BALB/c mice to pps 14. Mice received 0.5 µg human serum PF4 or mouse serum (0.2 ml 1:4 dilution) iv on day -3, at the same time as the tolerance-inducing injection of 0.2 µg pps 14 ip. They were challenged with an immunizing dose (25 µg) of pps 14 on day 0.

Effect of Mouse Serum and of Human PF4 on Immune Response to Pneumococcal Polysaccharide Type 3

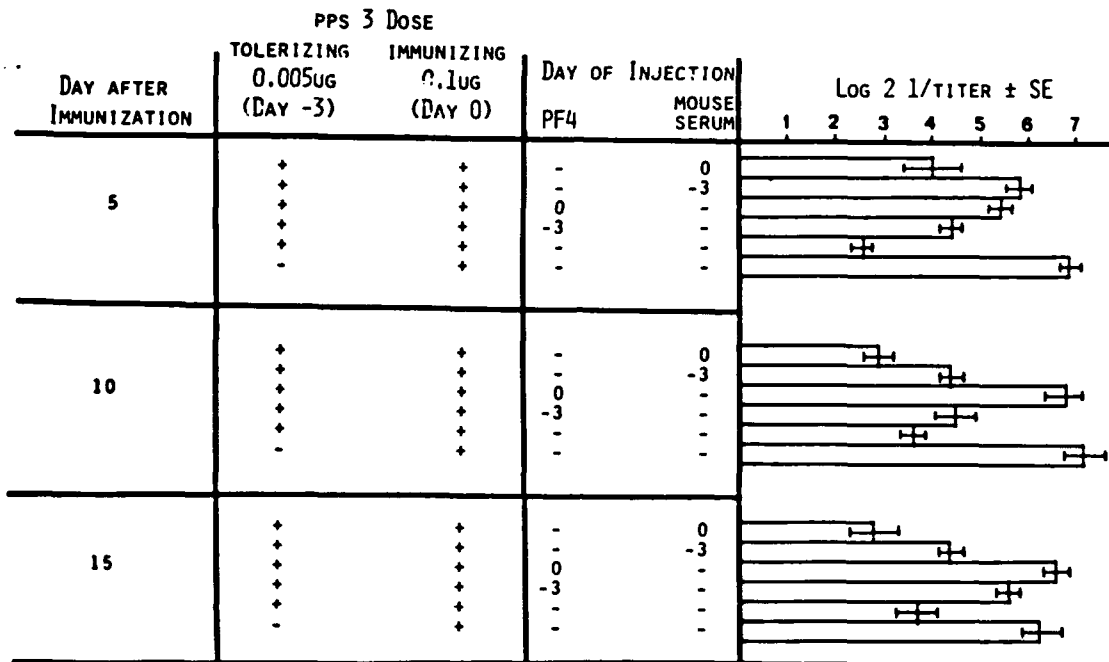| Day after Immunization	PPS 3 Dose		Day of Injection		Log 2 1/titer ± SE
	Tolerizing 0.005ug (Day -3)	Immunizing 0.1ug (Day 0)	PF4	Mouse Serum	1 2 3 4 5 6 7
5	+ + + + + -	+ + + + + +	- - 0 -3 - -	0 -3 - - - -	
10	+ + + + -	+ + + + +	- - 0 -3 -	0 -3 - - -	
15	+ + + + -	+ + + + +	- - 0 -3 -	0 -3 - - -	

Fig. 13: Prevention of low dose tolerance in BALB/c mice to pps 3. Mice received 0.5 µg human serum PF4 or mouse serum (0.2 ml 1:4 dilution) iv on day -3 at the same time as the tolerance-inducing injection of 0.005 µg pps 3 ip. They were challenged with an immunizing dose (0.1 µg) of pps 3 on day 0. Some mice received the PF4 or mouse serum on day 0.